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TECHNOLOGY TRANSFER CONFERENCE

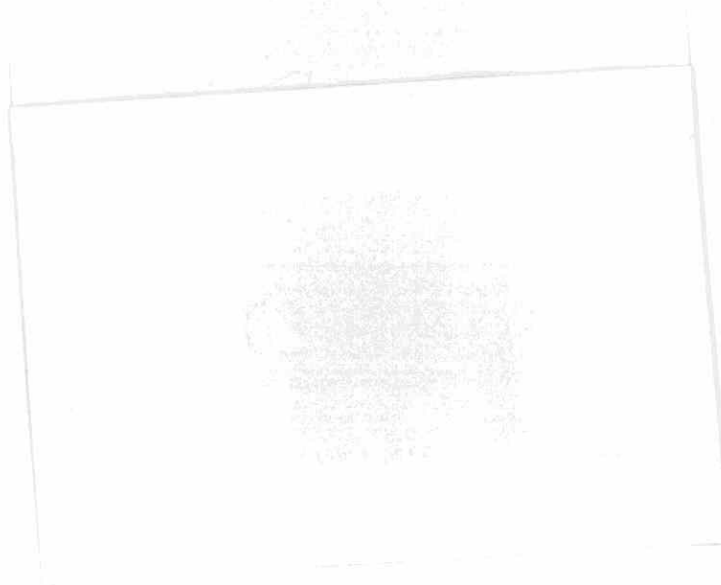
December 8 & 9, 1986

THE SHERATON CENTRE
TORONTO, ONTARIO,
CANADA

Part D
Analytical Methods

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PROCEEDINGS

TECHNOLOGY TRANSFER CONFERENCE

December 8 & 9, 1986

The Sheraton Centre

External Research Projects

PART D
ANALYTICAL METHODS

Organized by
Research Advisory Committee

Sponsored by
Corporate Resources Division
Ministry of the Environment
Ontario
CANADA

Goal Statement

"To achieve and maintain a quality of the environment - including air, water and land - that will protect human health and the ecosystem and will contribute to the well-being of the people of Ontario."

ACKNOWLEDGEMENTS

The Conference Committee would like to thank the speakers and organizers for their valuable contributions.

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INTRODUCTION

The Ontario Ministry of the Environment holds its annual Technology Transfer Conference to report and publicize the progress made on Ministry-funded projects. These studies are carried out in Ontario universities and by private research organizations.

The papers presented at the Technology Transfer Conference held in December 1986 are presented in Conference Proceedings. These Proceedings are divided into five parts corresponding to the conference sessions dealing with:

Part A: Air Quality Research ;
Part B: Water Quality Research;
Part C: Liquid & Solid Waste;
Part D: Analytical Methods; and
Part E: Environmental Economics.

This part is a collation of those papers presented at Session D of the Conference.

For further information on any of the projects, the reader is kindly referred to the Principal Investigators or to the Research Management Office at (416) 965-5788.

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TRENDS IN MULTIELEMENT ANALYSIS: THE ROLE OF
ICP/MS IN ENVIRONMENTAL APPLICATIONS

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NOVEMBER, 1986

FOR

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I BACKGROUND

This review attempts to look at trends in multi-element analytical instrumentation in major environmental laboratories, in terms of both the forces shaping these trends and the technical instrumentation capabilities, with particular emphasis on the emerging role of plasma/mass spectrometer systems.

It is always dangerous to attempt such reviews, not only because oversimplifying generalizations are involved, but also because of the pace of change. This latter is especially true in this instance because both the pressures on environmental laboratories and instrumentation capabilities are evolving rapidly.

In overview, the needs facing the management of major environmental laboratories everywhere are simultaneously:

- increased requirements for multi-element trace component analysis at ever decreasing concentrations, sometimes with determination of the chemical form of the trace elements as well
- massive increases in sample throughput requirements, which together with the multi-element requirement produces a tremendous pressure for development of the most cost-effective strategies on a per determination basis
- unimpeachable, legally defensible data

These world-wide needs are facing major industrial laboratories just as much as those of the regulatory agencies. A financial investment review of the instrumentation industry (Ref.1) discusses the driving forces behind a generally predicted expansion rate of 25-30% per year, for instruments answering these needs, as follows.

(1) New Legislation and its Enforcement

North American examples include the U.S. Resource Conservation and Recovery Act (RCRA), \$500 million of the Superfund for toxic waste analysis, and the Ontario MESA Project and Bill 309 on waste disposal. Industrial costs for compliance monitoring will be as large or larger than those of government agencies. Amendments to the U.S. Safe

Drinking Water Act are probable causes for increased monitoring of point source emission (factories) and non point (e.g., farmland). Acid rain related phenomena, with cross-boundary impacts in North America, Western Europe and Asia, are forcing legislation and measurements which are international in scope.

(2) Litigation and Liability

The enormity of toxic substance related legal settlements and/or abatement methodology costs, particularly in the U.S., but increasingly world-wide, is putting a premium on legally defensible unimpeachable analytic data, and plenty of it. This applies both on the industrial side in proving compliance and on the regulatory agency side, in documenting site emission.

(3) Emerging Public and Consumer Attitudes

An increasingly knowledgeable and pollution-conscious public creates a demand for information on trace toxic chemicals in food, air, water, foodstuff and manufactured goods.

(4) Process Control and Monitoring

The trend toward higher technology industrial development, such as the semiconductor and biotechnology sectors, is generating a concomitant need for approved trace chemical analysis, driven by impact awareness in both directions. For example, impurities in input water and air have costly adverse effects on integrated circuit manufacture and fine chemical production, both growth areas. Conversely, silicon valley companies have already spent over \$100 million on toxic chemical storage clean-up and workplace monitoring.

It seems clear that these driving forces are now being amplified by a very positive mutual feed-back effect. Available analytical instrument capabilities and the already available data from them are beginning to paint a clearer picture in many environmental, occupational and industrial areas internationally, and this ability to translate the trace chemical environment into a visible picture is a widely perceived technical reality. Termed the Rosetta stone effect, the demand for the analytical instrumentation to provide more and more translations of the complex chemical messages in the total planetary ecosphere is growing inexorably.

Much of the data which has had broad societal impact, such as the blood lead level in human population groups or the mercury in fish or in bottom sediments in river systems, has been obtained to date at natural bio-accumulator points in the total system. Increasingly, attention is turning to documentation of pathways on both a macro and micro scale, not only to obtain a better scientific understanding of the transport phenomena involved and hence true cause and effect, but also to provide unassailable documentation for political, legal, industrial or public action. For the analyst, pathways such as acid rain, rivers, aquifers, drinking water, urban air and dust, estuarial waters involve much lower concentrations of the trace elements of interest - typically sub parts per billion (or sub nanograms per gram) which not only push instrumentation capabilities to the present limits but equally demand of analytical laboratory operators unprecedented standards in sample collection, handling, laboratory cleanliness and verification of data.

There is increased awareness of the importance of knowing the chemical forms in which the element is present in the environmental sample. For example, tetra ethyl lead or alkyl mercury are not only orders-of-magnitude more toxic than the elemental forms, but also the transport and accumulation mechanisms are dramatically different. Thus another emerging challenge is to provide analytical capability for chemical form determination at the trace level.

The next section briefly reviews the present multielement analytical instrumentation situation, and particularly attempts to place the emerging role of plasma/mass spectrometer instruments into the perspective of the foregoing admittedly dangerous generalizations.

II MULTIELEMENTAL ANALYSIS INSTRUMENTS: THE PRESENT AND EMERGING PLAYERS

Since there have been a number of excellent recent reviews of specific instruments, it seems most appropriate to summarize briefly, citing from those reviews and focussing on the environmentally important aspects.

A well-equipped environmental multielement laboratory today will probably have as major instruments:

- energy and wavelength dispersive X-ray fluorescence spectrometers

- flame and/or graphite furnace atomic absorption optical spectrometers (AAS)
- plasma (likely inductively coupled) atomic emission spectrometers (ICP/MS)

and some will have, in addition to these x-ray and optical instruments, the relatively new

- inductively coupled plasma/mass spectrometers (ICP/MS)

Additionally, some laboratories may have access to neutron activation analysis capabilities. This is a powerful technique but not generally available and will not be covered in this review of instruments.

X-Ray Fluorescence Spectrometers

X-Ray instruments utilize a closed x-ray excitation source and a closed detector to provide both energy and wavelength dispersive data from which absolute amounts of various elements in the sample can be determined (Ref.2). First introduced commercially in the 1950's and 60's by instrument companies such as Philips, Siemens, Analytical Research Laboratories, Rigaku and Kevex, they are by now highly developed, reliable and stable instruments. Although relatively expensive, with modern computer-controlled acquisition, manipulation and presentation of data they rate highly on sample throughput and cost per determination scales, and provide reliable data.

Unfortunately, the physical limitations imposed by the brightness of available x-ray sources, the necessary source-sample-detector geometries, and detector sensitivities all place detection limits typically in the micrograms (or parts per million for a gram sample) range, and worse for low z elements. Because of the limited penetration power of the x-rays, they also tend to be limited to near-surface analysis, making them particularly well-suited for some forms of sample, such as filter-collected airborne dusts. With these limitations in mind however, they deserve careful consideration in planning environmental laboratory protocols for major sample processing campaigns, in that they provide reliable determinations of major and minor sample analytes, particularly useful in a pre-screening role before subsequent trace analysis by other instruments.

The precision and accuracy of the signals they produce are statistically limited, and since these instruments are relatively mature in their development cycle, there does not seem to be a reasonable expectation of major improvements in sensitivity and detection limits.

Flame and Furnace Atomic Absorption Instruments

Although they are not strictly speaking fully multielemental in practical terms, the acceptance and rapid growth in use of first flame and later furnace atomic absorption instruments has led to their use in many multielement environmental applications. The combination of reasonable capital costs and picogram-level detection limits (with furnace AA) for many elements is part of the reason. These instruments have EPA approval.

AAS instruments utilize an atomizer source (flame or furnace) to convert at least some of the sample into dissociated atoms in the source. Light from a radiation source for the element of interest is passed through the flame and is attenuated by resonant absorption proportional to the concentration (Ref.3). Commercial instruments are available from Varian, Perkin-Elmer, Thermo-Jarrel-Ash and other firms. Linear response ranges are not very large - typically approaching two orders-of-magnitude, so care must be taken with analyte loadings.

It is geometrically practical to provide up to six lamps for sequential multielement analysis. With the present highly developed automation of these flame AA instruments, 85 samples per hour can be analysed for six elements each, or about 500 determinations per hour. (Ref.4).

Flames are poor dissociators for the more refractory elements and the alkaline earths. Also, elements with their resonance lines in the far ultra violet such as the halogens, sulphur and phosphorous are not easily determined, with conventional commercial instruments.

Compared to flames, graphite furnaces provide up to 1000 times better detection limits. Although initially plagued with major interference effects, these have now been commercially developed, through better furnace technology, better photometric design and the elegant use of the Zeeman effect to reduce background interference. Both flame and furnace AA tend to suffer from chemical matrix problems related to volatilization and dissociation effects in the flame or furnace, while

the ICP optical systems discussed below have much less problems in this area but do have problems from the overlap of atomic emission spectra.

A simultaneous multielemental AA system using a continuum source, initially studied by O'Haver, termed SIMAAC, has been under laboratory development for several years (Ref.5), but is not commercially available. Based on graphite furnace atomization, even with the Xenon-lamp continuum source brightnesses presently available it offers superior detection limits for many elements, and seems less susceptible to cross-compound optical spectral interferences than the optical systems discussed below. Unfortunately, lamp brightnesses fall off drastically in the far ultraviolet so elements absorbing in this region are poorly detected.

Although research groups have studied the possibility of utilizing atomic fluorescence (rather than absorption) in the form of simultaneous multilamp, multielement furnace atomic fluorescence (Ref.6) no commercial instruments have yet appeared.

Plasma Emission Instruments

The most highly developed truly multielemental trace analysis systems today are based on measuring the optical emission spectra of atoms or ions either from a DC arc or from an induction coupled plasma tail-flame (ICP/AES). While DC arc-based systems are in wide-spread use in industrial laboratories, environmental laboratories have tended to utilize predominantly instruments based on induction coupled plasmas. Multiple detectors can be arrayed along the focal plane of the spectrometer for simultaneous determinations of up to 60 elements, utilizing optimally selected emission lines to help minimize cross-element spectral interferences. Background correction can be provided by scanning to obtain a measure of the background signal near the lines of interest. Alternatively, a fast scanning sequential monochromator mode may be used. Marketed by companies such as Perkin-Elmer, ARL, and Thermo-Jarrel-Ash, these systems are highly automated and computerized. 40 to 50 elements per minute or 3000 determinations per hour can be achieved on the Perkin-Elmer instrument. Ultimate detection limits are not as good as graphite furnace AA, typically being in the low to mid parts per billion range, with refractory metals, arsenic, lead, selenium and other heavy metals of particular interest environmentally being detected with least sensitivity.

At their present state of development these systems have a signal stability of better than 1% per hour. Determinations to 1% precision can be made, with perhaps 1% accuracy if recalibrated every 2 hours with a similar matrix calibration solution.

Plasma/Mass Spectrometer Instruments

The induction coupled plasma also can be used as a mass spectrometer ion source. Instead of utilizing optical emission or absorption from atoms and ions, elemental ions from the plasma tail flame are extracted into a mass spectrometer for analysis via a mass spectrum. In the six years since the first paper was published (Ref.7), there has been rapid growth in the technique with two commercial instruments introduced - by SCIEX® Division of MDS Health Group Limited in Canada in 1983 and V.G. Isotopes in England in 1984. Basically, this growth occurred because this approach seemed to offer three simultaneous advances over optical multielemental analysis instruments - improved detection limits over the complete periodic table of elements, a spectral simplicity which could in principle, vastly reduce spectral interference problems, and the intrinsic capability of providing isotopic abundance data along with multielemental analysis.

Reviews of ICP/MS were published in 1985 (Refs.8, 9), but there has been very considerable progress made in improving key performance parameters since then, which this review will attempt to summarize.

The basic principle of operation of an ICP/MS system can be understood from Fig. 1, and more detail is provided in Refs. 8 and 9. Most commonly, solutions to be analysed are sprayed into a conventional atmospheric pressure ICP using a nebulizer, but it is obvious that many alternative methodologies for sample introduction can be applied. Under typical sample loading and powers, most elements in the periodic table are highly ionized in the ICP.

Gasdynamic beam techniques utilizing a two stage expansion through conical orifices then extract the center line flow of the plasma tail flame into a vacuum region housing a quadrupole ion filter, which can be rapidly scanned electronically to provide a complete mass spectrum or can be used in a rapid peak-hopping mode to monitor only selected elemental ions.

The usual ion sampling position in the plasma tail flame is approximately the same as the viewing region in the optical systems. The instantaneous signal is proportional to the centre line ion flux crossing the sampling orifice rather than the instantaneous integrated concentration of atoms and ions (depending on the emission lines selected) in the viewing region of an optical system, but these signals are very closely related. This correlation has been closely documented in a paper by Gillson (Ref.11), using simultaneous optical and mass spectrometric sampling of the plasma tail-flame. Thus, while the basic spectral line interference situation for ICP/MS is vastly simpler than for ICP/AES, and detection limits better, the chemical plasma matrix interference effects should be fundamentally almost identical for the two systems.

Many studies have shown that the ICP is relatively free from chemical matrix interference effects compared to flame and furnace AA, due to the high degree of dissociation and ionization of the analytes, an important attribute for achieving wide linear dynamic response range and true multielement analysis capability. However, high plasma loadings either from large nebulizer liquid flow rates or high inorganic matrix content (such as high salt content) can load the plasma so that the instrument sensitivity for individual elements can be affected. This applies to both ICP/AES and ICP/MS systems.

Under typical ICP conditions of about 8000°K and 1 atm pressure, the sample will be a dense plasma containing about 10^{15} total ions and electrons per cm^3 , dominated by the reagent argon carrier ions and with ionic populations of the analytes proportional to their concentrations. It turns out that in this plasma there will also be, at very much lower levels, unwanted but inescapably present amounts of trace molecular ion species of some analytes (particularly refractory oxides) and also trace but measurable amounts of some doubly ionized species (which appear at half the nominal mass). This problem, which is primarily one of spectral interference, is however generally orders-of-magnitude less than that existing in instruments based on optical spectral emission.

The key to developing the full analytical potential of ICP/MS lay in successfully extracting this plasma through the sampling interface.

It is now clear that it is feasible to sample and retain the ionic population representative of the undisturbed plasma, because the rate of expansion is so rapid that it effectively freezes the ion population, so long as unwanted shock waves, boundary layer cooling effects and spurious electrical discharges are effectively eliminated from the central plasma expansion. This plasma sampling system was first developed by Douglas and French (Ref.10) and is incorporated in the SCIEX® ELAN® ICP/MS system.

III PERFORMANCE PARAMETER COMPARISONS

Because the ICP/MS commercial systems are in a relatively early phase of their development cycle compared to the other multielemental analysis instruments, further improvement in important operational parameters can be reasonably expected. The following discussion should be regarded as a snapshot taken at the time of writing.

Detection Limits

Detection limits in instruments are like horsepower in sports cars - often essential for the desired performance, difficult if not to say dangerous to utilize fully except in the hands of an expert, and not always the key determinant of user satisfaction in the long run.

Table 1, with portions adapted from Ref. 4, compares the present detection limits for ICP/MS (data from Ref. 12) with the other systems. As noted in Ref. 4, the furnace AA instrument provides an absolute-amount-limited response whereas the ICP (with nebulizer sample introduction) provides a concentration-limited response, and the comparison in Table 1 is made by assuming a 20 microlitre sample introduced into the furnace. This provides the picogram scale on the left. But ICP based instruments, optical or mass spectrometric, are not limited to nebulizer sample introduction. As has been recently demonstrated, the use of an electrothermal vaporizer (Ref. 13), or direct insertion probe (Ref. 14) in combination with ICP/MS can equally provide sub-nanogram level detection limits. This will prove to be of importance in many sample limited environmental studies.

The ICP/MS data show generally one to two orders of magnitude better detection limit advantage over ICP/AES, rivalling furnace AAS for many elements. Even at the present stage of development, there is particular detection limit advantage for many environmentally important

elements, such as the transition metals and rare earths which are difficult to determine by other means, but of increasing importance in studying the environmental pathways for oil and coal combustion products.

The ICP/MS detection limits shown in Table 1 are those obtainable under normal operating conditions, with nebulizer uptake rate at 2 ml/min, ICP power at 1200 watts and total solid content of the analyte solution not greater than 0.2%. It is possible to improve these detection limits by factors of 5 to 10 by resorting to higher nebulizer flows, but the increased plasma loading will incur worsened matrix effects.

Response Range

While the linear response range for flame or furnace AA spans two orders of magnitude, and ICP/AES reaches between four and five, the usable response curves for ICP/MS (Fig. 2) span over 5 orders-of-magnitude. This is primarily because, with presently achieved ion extraction efficiency, the low end detection limit is generally close to the limit set by counting statistics, rather than being set by the ability of the instrument to extract the signal from a continuum or other interfering background. (The true noise background is a few counts per second). The upper limit comes from electron multiplier saturation at over 10^6 counts/sec.

This wide dynamic range helps both in initial semi-quantitative sample screening functions, and in providing better multielement analyses when trace and minor components are present in widely varying concentrations.

Signal Stability

The signal stability of an ICP/MS instrument depends obviously on the stability of operation of all parts in the system - nebulizer, plasma torch, ion extraction interface, ion quadrupole filter, lenses and detection system stability. In comparison with ICP/AES systems, the key differential area in determining relative stability is the ion sampling interface, since modern ion filtering and detection systems have very good stability. The stability possible at the present state of development of one commercial system, the SCIEX® ELAN®, has been reviewed by Douglas (Ref. 15). Taken from this reference, Fig. 3 shows

the raw ion count rates for elements, each present at 100 nanograms per millilitre, measured over 13 hours. It is seen that there is a drift of 1-2% per hour. Most elements drift in the same direction which means that an internal standardization can be readily applied to correct much of this drift. Fig. 4 shows that, with the addition of one mid mass range internal standard (in this case rhenium) a stability of $\pm 1\%$ over six hours is obtained. This compares well with good ICP/AES systems, leading to the important conclusion that ion sampling interfacing can be achieved without introducing additional drift, for solutions of this dilution.

However, for a refractory matrix of salt content of 0.1% or greater an additional source of drift can be encountered which is apparently due to gradual salt deposition on the sampler or skimmer cones. Fig. 5 shows the stability obtained for 6 elements at 100 nanograms/millilitre in 2000 parts per million uranium salt solution. Despite the high salt content, signals decreased by less than 10% over 8 hours.

In the reviewer's opinion, this salting artifact should not be viewed as a fundamental limitation of the ICP/MS system. Rather, it is encouraging that this level of stability has been achieved so soon in the overall development. It is quite possible, with the development of more refractory skimmers and samplers, that operation at higher temperature (reduced cooling) will in future eliminate this salting problem. This could be a minor modification to existing systems.

Matrix Effects

These remarks apply equally to a second phenomena, also discussed in Ref. 15. At the present stage of development of ICP/MS technology, there can be signal suppressions varying between 0 to 50% for salt content of the order of 0.1% (1000 ppm). This suppression effect, which is more severe than that observed in ICP/AES instruments, is generally worse at higher plasma sample loadings and is mitigated with light loadings. The cross-element interferences are generally mass dependent.

Higher salt content than 0.1% can be run provided the analyst uses careful matched matrix calibration to compensate. The method of standard additions is very helpful. However, limiting the total solid content to 0.1% is generally recommended as more straight forward at

present. While this is lower than the tolerable levels in ICP/AES, the superior detection limits offer compensation.

This effect, obviously greater than the matrix effects in the plasma flame well-documented by ICP/AES users, is by inference related to the ion sampling process, and is not well understood at present. Again, there are no reasons to expect that this is fundamental, and improvement can be reasonably expected as the basis of the phenomena becomes better understood.

In the meantime however, it has already been demonstrated by McLaren, Beauchemin and Berman (Ref. 26) that the isotope dilution technique (see below) available with ICP/MS can be used successfully to eliminate matrix suppression errors and thus permit analysis of more concentrated solutions.

Possible Spectral Interferences

There can be occasional spectral interference in ICP/MS from isobaric ions which can cause difficulties with specific ions. As mentioned above, the plasma at 8000°K can contain small amounts of doubly charged ions and refractory metal oxides, depending on the sample matrix (Ref. 16). Also, if acids other than nitric are used in sample work-up, molecular ions of the acid species have been observed and can interfere. A list of potential interferences has been provided by Vaughn and Horlick (Ref. 17). Besides nitric acid digestion, McLaren and co-workers have discussed useful alternate sample preparation processes developed to minimize ICP/MS molecular ion interferences (Ref. 18). In general, these spectral interference effects are small compared to the situation in ICP/AES instruments, but they can be troublesome in some specific element/matrix situations.

Isotope Ratio Determination Capability

This capability, effectively unique to mass-spectrometer-based instruments, depends on several instrument parameters. The precision achievable for each isotope is determined in the limit by counting statistics, but sometimes more dominant noise sources can be present such as plasma flicker due to nebulizer spray droplet statistics. Accuracy can be affected by the abundance ratio capability, a spectral resolution number which describes the degree to which the tail of adjacent mass peaks overlap into the central region of the ion being

measured. This abundance ratio is less than 10^{-6} (Ref.19), meaning that an isotope can be determined even in a matrix which presents adjacent mass ions one million times in excess.

Data contained in Ref. 15 illustrate the achievable accuracies possible at present using peak hopping to help average out plasma flicker noise. With silver at 1.0 microgram/millilitre, the $^{109}\text{Ag}^+ / ^{107}\text{Ag}^+$ ratio was determined to be $\pm 0.06\%$. This is a favourable case because the isotope abundance ratio is close to unity. For a less favourable case, strontium $^{87}\text{Sr}^+ / ^{88}\text{Sr}^+$, the data indicate .08% precision was achieved for a ratio of .086. An error of 1 to 2% compared to the actual ratio comes from a well-known mass discrimination bias across the mass range; when a correction factor is applied by calibrating with a certified isotope ratio solution, the determined ratios in test cases agree with known values to within the uncertainty of the experiment.

While these precisions do not match those obtainable on mass spectrometers designed solely for isotopic ratio determinations, they are nevertheless very much in the range that can be used in several ways to immense advantage in environmental work by permitting:

- (1) the isotope dilution technique to be used to provide the most fundamental guarantee possible of the correctness of the absolute concentration measurements
- (2) isotope ratios to be used in assisting in pollution source identification
- (3) isotopically labelled isotope tracers to be used in studies of pollutant pathways, particularly in biological situations.

IV THE GROWING ROLE OF ICP/MS: ENVIRONMENTAL EXAMPLES

The driving forces on environmental needs for sample analyses previously discussed, combined with the mix of performance parameters achieved in the ICP/MS instruments to date suggest a rapidly growing role for these instruments in environmental laboratories, and an equally rapid growth in the volume of data produced. However, it is important to recall that the improvement in detection limits achieved with graphite furnace led to many papers in which the data were later

found to be suspect. Claude Veillon vividly describes this situation in the story of trace chromium in biological samples (Ref. 20). With furnace AA instrumentation capable of three orders of magnitude better detection limits than previously available, standards of laboratory cleanliness and knowledge of all possible sources of trace contamination (or trace analyte loss) were not always equal to the task. Veillon suggests a "useful paranoia" in suspecting and checking every step of the analytical procedure - sample containers, transfer tubing, stopcocks, syringes, dilution and standard solutions, laboratory airborne dust, etc. While nothing is more crucial than the skill and insight of the analyst, the combination of similar detection limits together with isotopic dilution capability in the one ICP/MS instrument is a powerful help.

Isotopic dilution applied at the time the sample is initially collected also provides the best protection against error due to trace element loss in all subsequent sample handling (since these changes affect equally the unknown and the isotopically labelled spike) or changes in instrumental overall sensitivity. However, it cannot compensate for contamination, whether the source is leached from a container or from a particle of dust from the laboratory air. It should also be noted that errors can sometimes arise if the spike is not either introduced in or subsequently fully converted (equilibrated) to the same compound form as in the sample. Once again, the skill of the analyst remains the most essential ingredient.

These points are well illustrated in the recent paper by Beauchemin, McLaren et. al., (Ref. 21), on determination of trace metals in a river water reference material by ICP/MS. Quoting their conclusions directly

"The rapid sequential multielement detection capabilities of ICP/MS combined with its good detection power give their technique enormous potential for the direct trace analysis of aqueous samples. It was indeed possible to determine fifteen elements directly in a riverine water reference material, and five more elements after a 20 to 50 fold preconcentration. This work has shown that, although analyses with external calibrations are usually more subject to errors arising from ionization interferences (caused by concomitant elements) unless the matrix of the standard solutions used to calibrate closely matches that of the samples, they can nevertheless be used

rather efficiently in many cases, provided that an adequate correction for drift is made. The method of standard additions provides, in general, more accurate results than external calibrations as it compensates for ionization interferences. Finally, the isotope dilution technique gives by far the most accurate and precise results, but it requires two isotopes free of spectroscopic interferences, and equilibration of the spike isotope with the analyte must be achieved in order to assure their identical behaviour during any sample treatment prior to analysis."

The growing importance of ICP/MS to water sample analysis is further illustrated by the work of Taylor and Garbarino (Ref. 22) on setting up isotopic dilution ICP/MS for a program in measurement of trace metals in U.S. aquifers.

The Rosetta stone effect - the existence of trace chemical data which in turn creates a situation demanding more data - has been mentioned. Lead in the environment provides a classic example. Boeckx (Ref. 23) quotes data which show that lead blood levels in urban dwellers in the U.S. dropped from 15 to 10 micrograms per decilitre between 1977 and 1980 simultaneously with a similar percentage drop in the total tonnage of lead used in automotive gasoline. (Measurable toxic effects begin to appear in children with blood levels in excess of 10 micrograms per decilitre). While cause and effect on a large scale are clearly established, the relationships in specific local situations involving multiple lead sources, such as battery factories, old paint on buildings, and lead in old plumbing pipes can be much more complex. Because the ICP/MS can determine lead at trace levels and measure its isotope ratio to help indicate the source of lead, Hinners of EPA Las Vegas has embarked on a demonstration study using ICP/MS which has shown that the sources of lead can be distinguished by this method. (Ref.24). Similar work has been initiated by Sturges (Ref.25) for lead isotopes in airborne samples in Ontario.

Another paper by McLaren and co-workers (Ref.26) demonstrates the accuracy and precision achievable using isotopic dilution ICP/MS in multielement trace analysis of marine sediments of geological interest. Table II from Ref. (26) indicates as noted in this reference that "the detection power of ICP/MS makes possible the determination of many elements for which few, if any, reliable values have been reported for geological and environmental reference materials. This increases the

importance of isotopic dilution techniques in assuring the accuracy of results obtained using other calibration strategies."

Application of ICP/MS to the study of acid rain sources, transport and effect has already begun in Ontario (Ref.27), and will be described in more detail elsewhere in this Conference Proceedings. Boomer and other researchers in the Ontario Ministry of the Environment are developing the protocols for a large-scale study of acid rain. One aspect is to provide data to help in understanding the impact of acid rain on trees, by analysing not only the trace pollutant content of the rain fall onto the forest canopy but also the altered trace concentrations on the leaf canopy, the trunk stem and in the soil and root system. Another aspect is to permit multivariant analysis to be applied in receptor modelling studies. Multielement trace analysis of received rainfall at various sites, which can contain varying ratios of pollutants injected from multiple sources, can in principle be unscrambled to determine the contribution from each source. The characteristic trace element ratios for each source will be used, but in the long run the source-specific isotopic ratios of each trace element will be of invaluable assistance in assigning the burden of responsibility to each source.

There are many other examples of potential large scale environmental application of ICP/MS under consideration, including particularly drinking water analysis. Many of these will require, as is appropriate, extensive developmental testing before receiving regulatory agency approval. It is already clear however, as these early examples perhaps suggest, that ICP/MS will play a large role in this area in future.

TRENDS, INCLUDING THE CARE AND FEEDING OF SAMPLES

As noted, the increased detection limit capability of ICP/MS brings concomitant need for care in sample collection, handling and work-up. But the sheer volume of samples, together with the diversity of sample forms and matrices of environmental importance, is pushing development in the feeding of samples - more versatility, convenience and efficiency in the methodologies which feed the samples into the instrument. Velmer Fassel (Ref. 28) believes that there will be further innovations and improvements in sample introduction techniques beneficial to all ICP-based instruments, including improved ultrasonic nebulization with aerosol desolvation, direct injection combined with

hydride formation, ICP/MS utilized as an element - specific detector for liquid and gas chromatographic effluents, and direct aerosol injection from solids. This reviewer concurs, and would even extend the list, for there are many examples of this trend in the recent literature. First, the electrothermal vaporizer (ETV) has been combined with ICP/MS by Park et al. (Ref.13) in an improved geometry which provided over 80% transfer efficiency of the microsample into the ICP torch and indicated nanogram per gram range detection limits. In an equally promising alternate approach Boomer and Powell (Ref.14) have reported excellent results for microsamples inserted directly into the plasma on a refractory metal loop probe. Ultimately, ease of automation and flexibility of operation to permit staged drying, ashing and chemical matrix modification as desirable will be important.

The use of laser surface ablation combined with ICP/MS has been reported by Arrowsmith (Ref.29). Indicated detection limits are in the low nanogram per gram range. Besides the excellent sensitivity of this approach, it is noteworthy that, in an operating mode in which the laser is free-running and is rastered across the sample face, it offers the potential for direct homogenized multielement determination for the sample face, without the need for intermediate sample dissolution and work-up. This appears particularly attractive for filter-collected samples, for example.

Of particular interest to the biologically oriented aspects of environmental studies, the first papers reporting on gas chromatographic interfacing with ICP/MS have appeared. Van Loon et. al., (Ref.30) have directly connected a gas chromatograph to the ICP torch and using three pentylated tin compounds have reported results which suggest that for compounds which can be derivatized into a form suitable for the gas chromatograph, heavy metal specification of organic samples shall be feasible with high picogram range detection limits. In another study by Chong and Houk (Ref.31), organic-compound-specific elemental determinations of some common elements, including their isotopic ratios were made by GC/ICP/MS for mixtures of alcohols, aromatic esters and phosphorous acid esters. Additionally, Thompson and Houk have reported the first results with reverse phase liquid chromatography coupled to ICP/MS (Ref.32) providing element and isotope specific data. These demonstrations are of particular interest to future environmental studies such as pollutant uptake in plants using isotopically labelled tracers.

This trend toward improvement and diversification of sample introduction methodologies would not be complete without mentioning the steady move toward robotics and automation applied sample handling, preparation, and introduction into the instrument. When this is combined with an integrated approach which combines the output of various instruments for data acquisition, manipulation and reporting purposes, major advances in both the sample volume throughput and in cost reduction on a per determination basis can be expected.

In summary, the trends for major environmental laboratories suggested by this reviewer are:

- a growing role for the ICP/MS instrument, both as a stand-alone instrument and integrated with other instruments in a best-available-absolute-calibration role due to its demonstrated combination of attributes - high sample volume throughput, excellent detection limits, true multielement capability, and the precision and accuracy achievable, particularly with the isotopic ratio measurement capability.
- a challenge to the environmental analyst to use all possible analytical skills, particularly care in sample handling and in improving operating conditions of environmental laboratories to the level demanded by instrumental detection limits exceeding parts per billion (nanograms per gram).
- an improvement and diversification of sample introduction methodologies, combined with increased automation computerization and integration of various laboratory instruments.

Applied wisely, these capabilities should provide the key to the cost-effective provision of the truly immense amounts of reliable environmental data which will be demanded in future.

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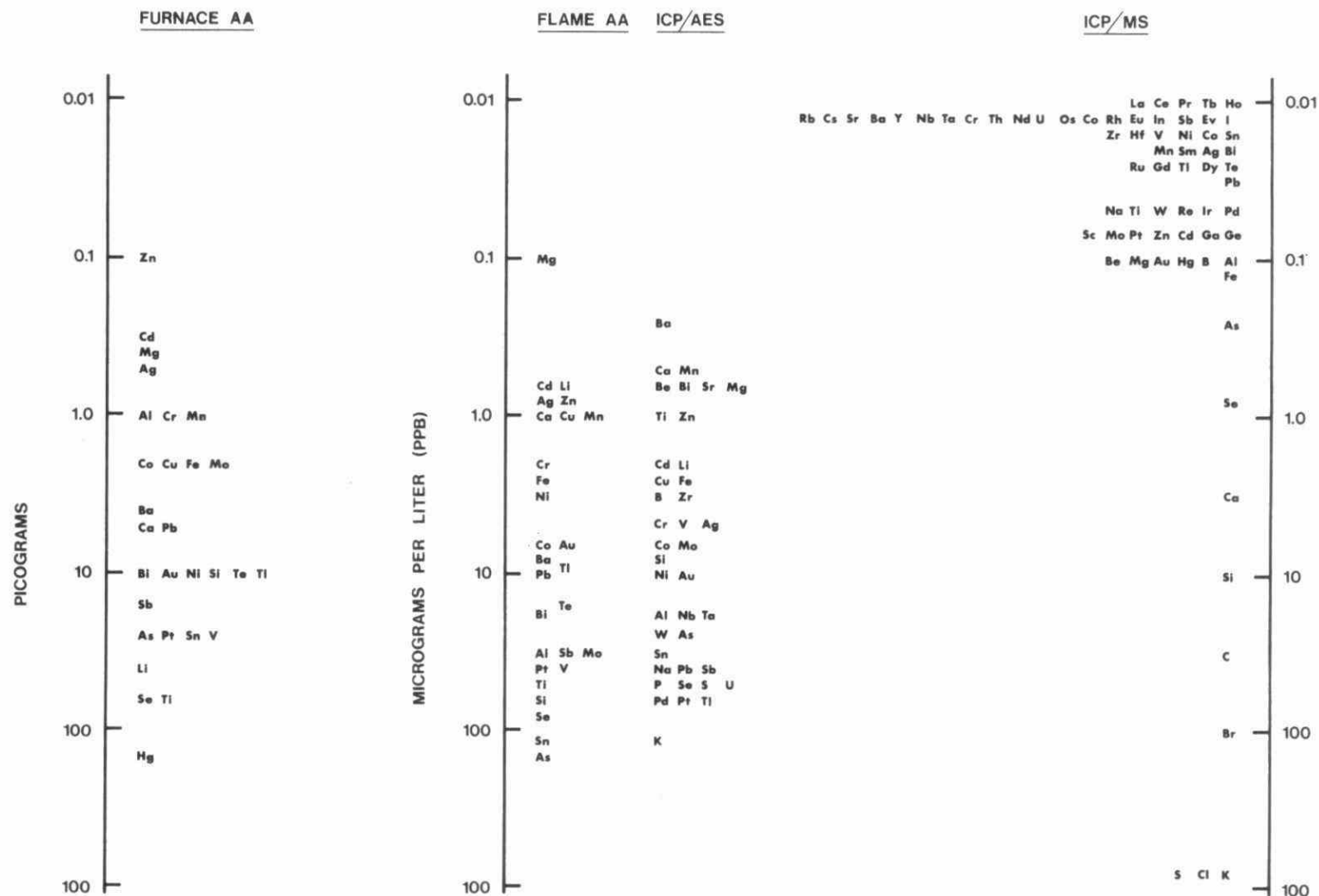


TABLE I
(from Ref. 4 & 12)

COMPARISON OF DETECTION LIMITS

TABLE II
ISOTOPE DILUTION ANALYSIS OF MARINE SEDIMENT REFERENCE MATERIALS

(from Ref. 26)

Element	MESS-1		BCSS-1	
	Found ¹	Accepted	Found ¹	Accepted
Cr	55.2 ± 0.8	71 ± 11	95.0 ± 1.2	123 ± 14
Ni	26.3 ± 0.3	29.5 ± 2.7	53.5 ± 0.6	55.3 ± 3.6
Zn	171 ± 2	191 ± 17	85 ± 4	119 ± 12
Sr	94 ± 1	(89) ⁵	108 ± 2	(96) ⁵
Mo	2.2 ± 0.1	(2.2 ± 0.3) ²	2.03 ± 0.04	(1.9 ± 0.2) ²
Cd	0.40 ± 0.03	0.59 ± 0.10	--	0.25 ± 0.04
Sn	3.8 ± 0.2	(3.4 ± 0.2) ⁴	1.75 ± 0.06	(1.8 ± 0.2) ⁴
Sb	0.67 ± 0.04	0.73 ± 0.08	0.47 ± 0.05	0.59 ± 0.06
Tl	0.74 ± 0.02	(0.70 ± 0.03) ²	0.65 ± 0.03	(0.60 ± 0.05) ²
Pb	33.1 ± 0.7	34.0 ± 6.1	22.9 ± 0.5	22.7 ± 3.4
U	4.1 ± 0.4	(4.2 ± 0.3) ³	2.7 ± 0.2	(2.7 ± 0.2) ³

1. Results in ug/g; precision expressed as the standard deviation (n=16).
2. Isotope dilution spark source mass spectrography with preconcentration by electrodeposition.
3. Isotope dilution spare source mass spectrography.
4. Hydride generation graphite furnace atomic absorption spectrometry. (14).
5. Flame atomic absorption spectrometry.

FIG. 1

ICP/MS SCHEMATIC

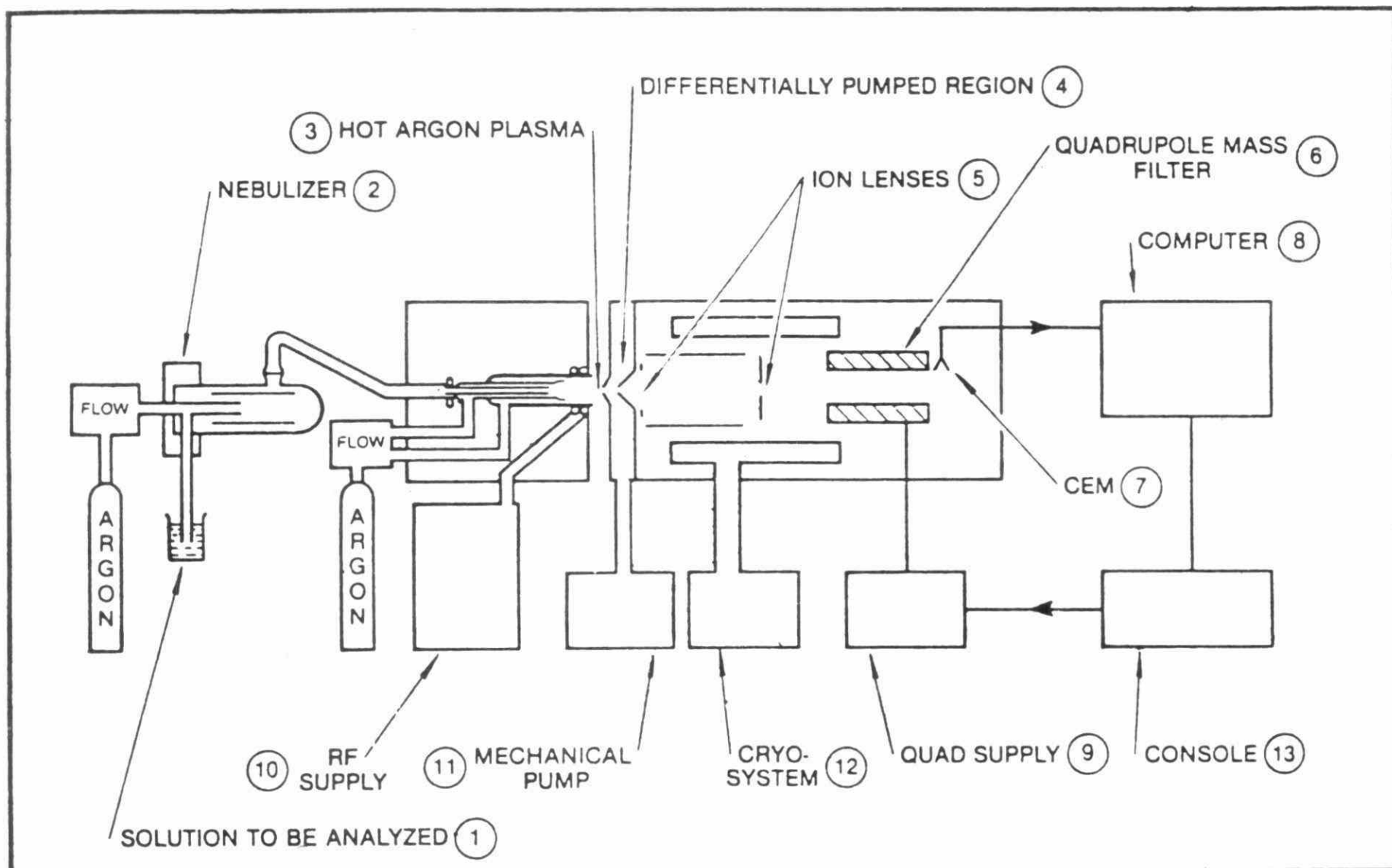
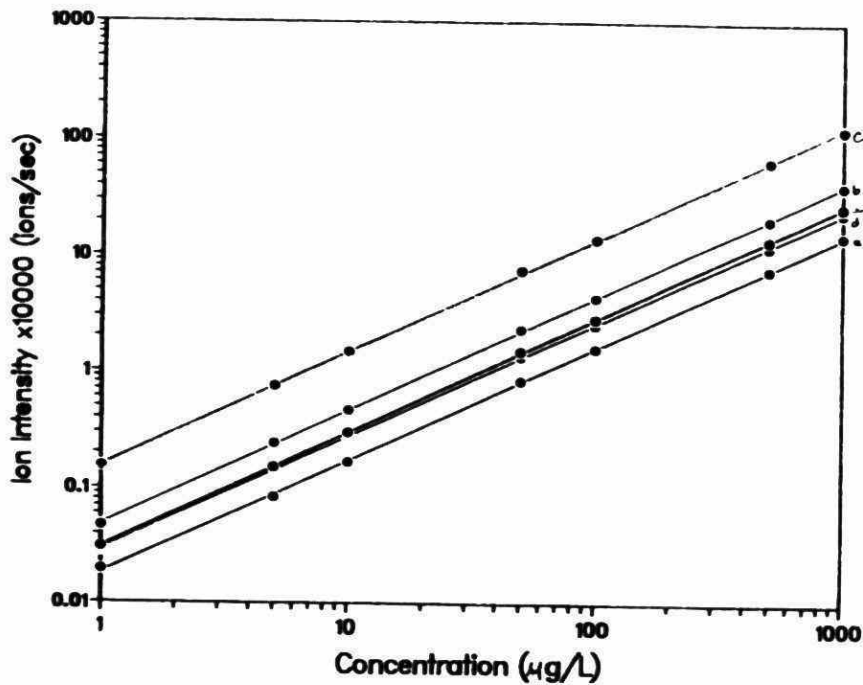


FIG. 2

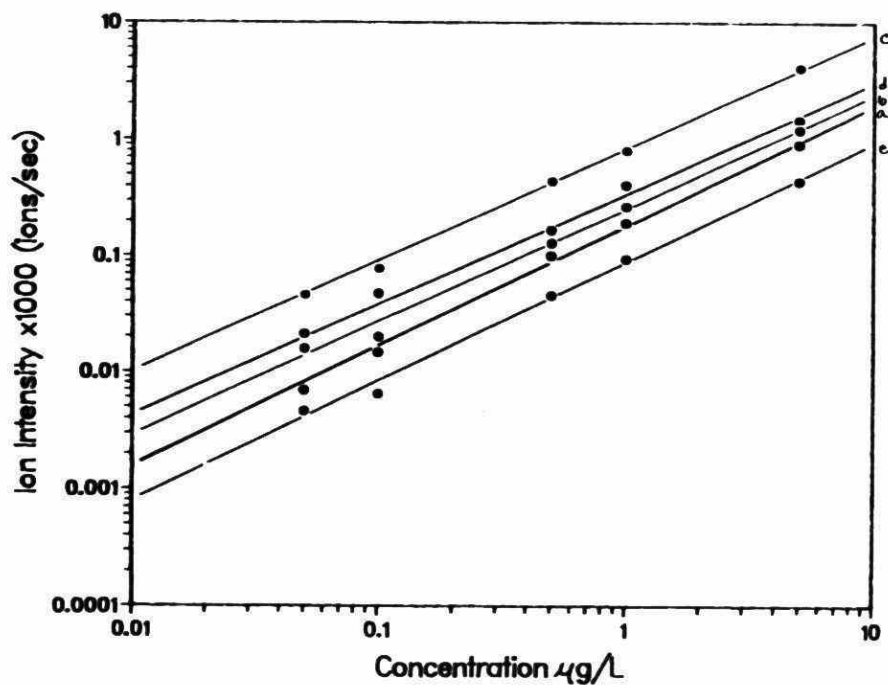
TYP. ELAN CALIBRATION CURVES

CALIBRATION CURVES



Ref. H. Taylor
& J. Gabarino
1986 Winter
Conference on
Plasma Spectro-
chemistry paper #7

CALIBRATION CURVES



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chemistry paper #7

FIG. 3 (from ref.15)

ELAN SIGNAL STABILITY

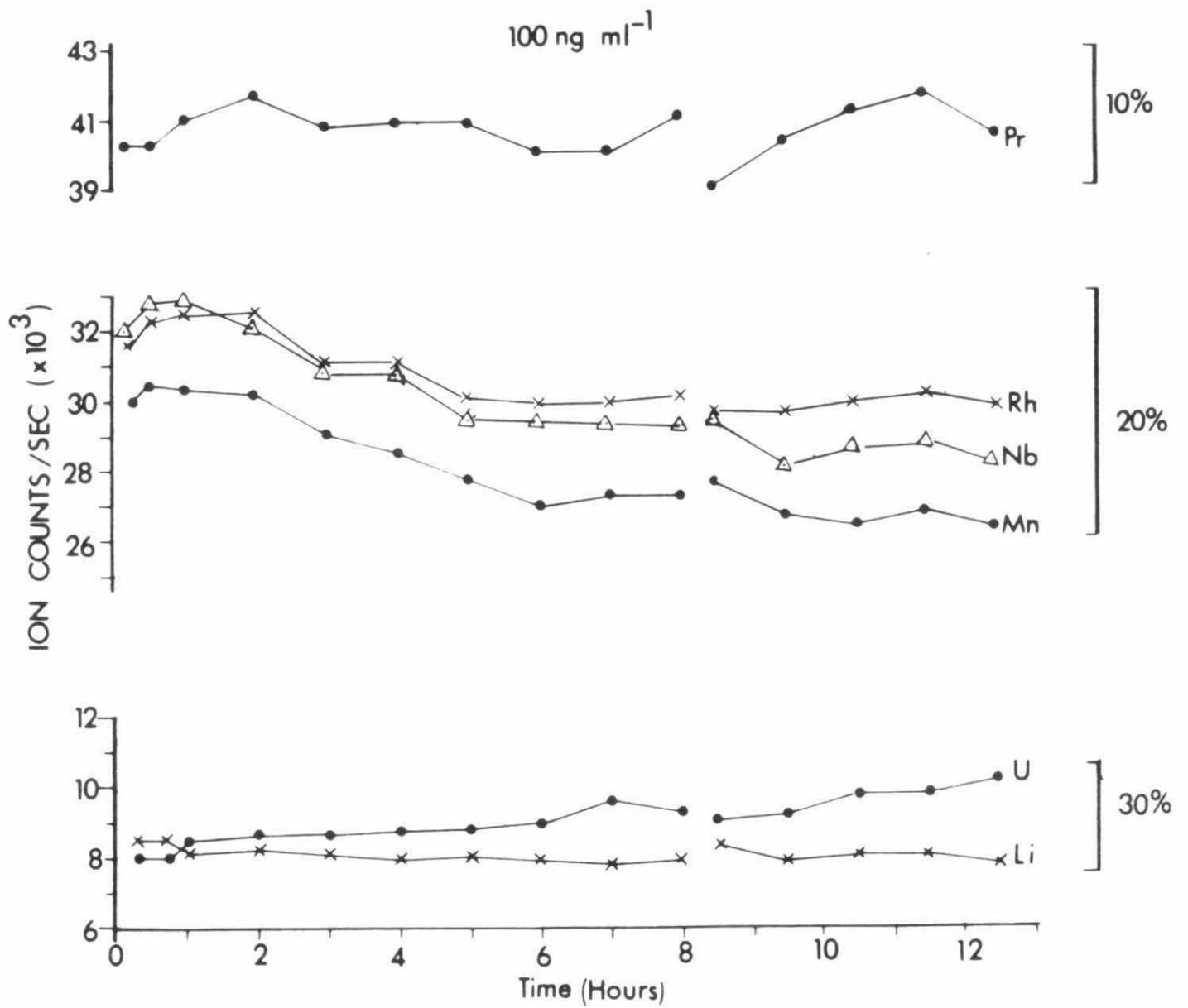


FIG. 4 (from ref. 15)

STABILITY WITH AN INTERNAL STANDARD (Nb)

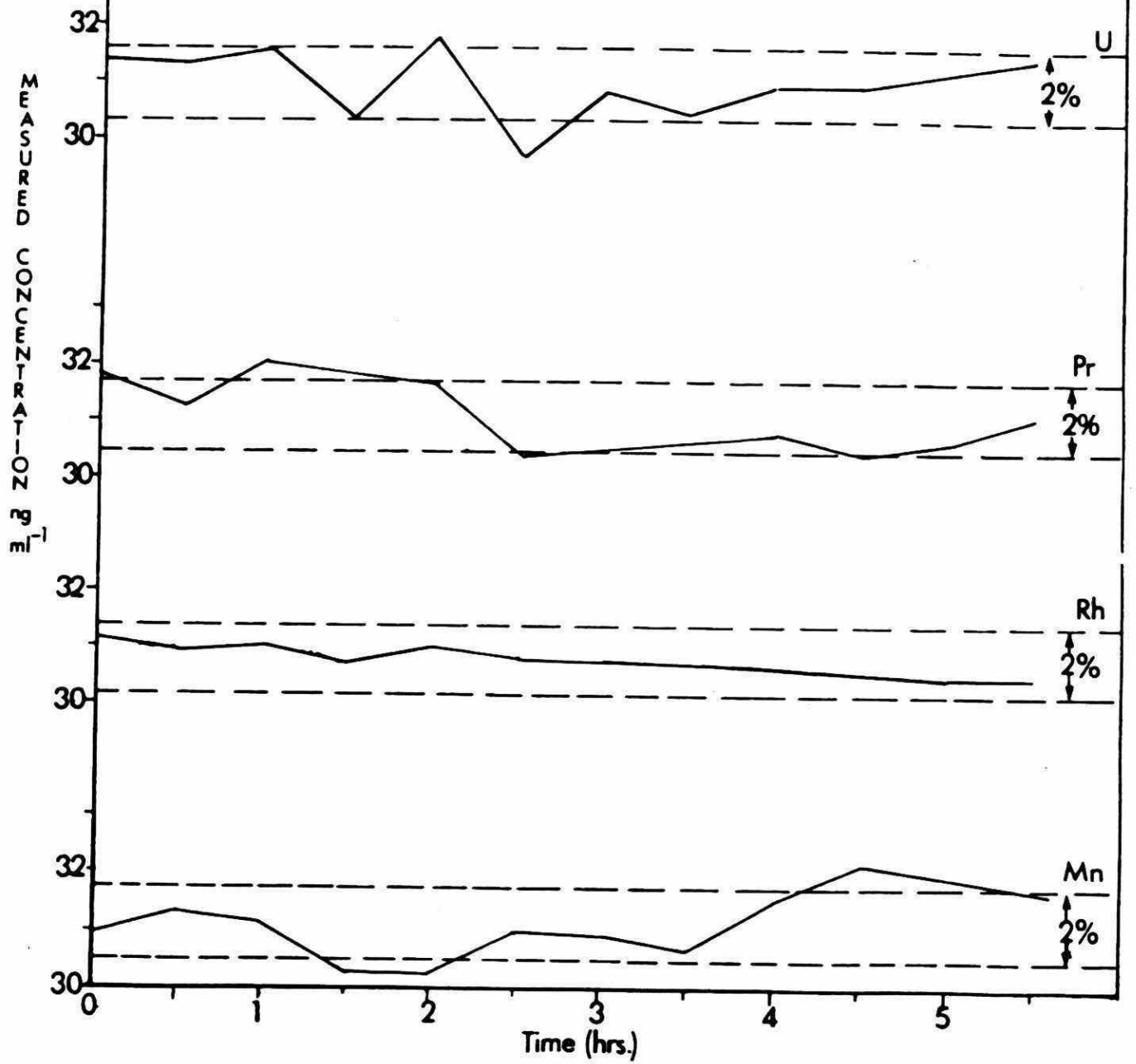
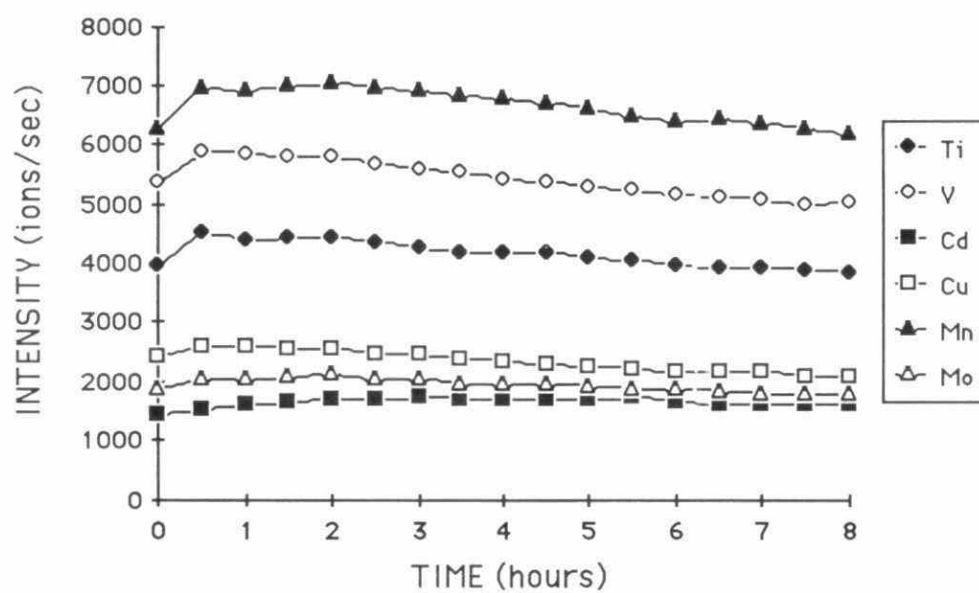


FIG. 5 (from ref.15)

STABILITY IN THE PRESENCE OF 2000 PPM URANIUM



DETECTION OF HYDROXYLATED NITRO-POLYNUCLEAR AROMATIC
HYDROCARBONS AND KETONES IN AN AMBIENT AIR PARTICULATE
EXTRACT USING BIOASSAY-DIRECTED FRACTIONATION

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NOTE: Submitted to Environmental Science and Technology.

Abstract

The identification of hydroxylated nitro-polynuclear aromatic hydrocarbons (OH-NO₂-PAHs) and hydroxylated nitro-polynuclear aromatic ketones (OH-NO₂-PAKs) in an ambient air particulate extract by electron impact (EI) and negative chemical ionization (NCI) high resolution gas chromatography/mass spectrometry (HRGC/MS) analyses is presented here. Bioassay-directed fractionation through three sequential separations led to detection of these OH-NO₂-PAHs/PAKs in a highly mutagenic and substantially simplified subfraction of the extract. The compound class identification was based on comparison of both EI and NCI mass spectra of reference OH-NO₂-pyrenes. The HRGC/MS retention values for nine OH-NO₂-pyrene isomers indicated that one of the compounds detected may be an OH-NO₂-fluoranthene isomer. Quantification of this compound class by NCI HRGC/MS, on-column injection, full scan acquisition, is demonstrated with sensitivity to 0.5 ng/μL.

INTRODUCTION

Urban air particles contain extractable organic matter which has both mutagenic (1) and carcinogenic (2,3) activity. Detection of the specific compounds responsible for the mutagenic activity has been limited by the complexity of these extracts and the presence of highly polar components which have been difficult to identify. To facilitate identifications, these extracts must be separated into substantially less complex fractions and analyzed with newer analytical techniques. Fractionation followed by microbial mutagenicity studies has been utilized for identifying known or suspected mutagens in cigarette smoke (4), kerosene flames (5), synfuels (6), xerographic toners (7), and diesel exhaust emissions (8,9). Bioassay-directed fractionation has been proposed (10) as the most cost and time effective approach for identification of mutagenic compounds in complex mixtures by directing the analytical and identification research effort toward only significantly mutagenic fractions. This approach should be especially useful for the detection of heretofore unknown constituents of the matrix since the methods are not directed to, or developed for, specific compounds or compound classes.

Application of this approach to ambient air particulate extracts has to date been unsuccessful in identifying major mutagenic components (11,12,13). This result seems to occur because the mixtures are substantially more complex than specific emission sources (e.g., kerosene flames) or because a major portion of the mutagenicity appears in fractions which are difficult to analyze such as the acidic and polar neutral fractions. This paper reports tentative identification of potentially mutagenic hydroxylated nitro-polynuclear aromatic hydrocarbons (OH-NO₂-PAHs) and hydroxylated nitro-polynuclear aromatic ketones (OH-NO₂-PAKs) in both a polar neutral fraction and acidic fraction of an ambient air particulate extract using bioassay-directed fractionation.

EXPERIMENTAL METHODS

Collection, Extraction, Fractionation

The air particulate sample used in this study was collected in the Philadelphia area over a two week period using a Massive Air Volume Sampler (MAVS). The operation of the MAVS has been described elsewhere in detail (14). Only particles $<1.7 \mu\text{m}$, collected on Stage III of the MAVS, were used for this study.

A 49.1 g sample of the air particles was extracted using a Soxhlet apparatus under yellow fluorescent lights for 24 hours with a 300 mL volume of methylene chloride and then for 6 hours with a second 300 mL volume of methylene chloride (Burdick and Jackson, Distilled-in-Glass quality).

An overview of the total fractionation scheme which resulted in analysis of one mutagenic subfraction is shown in Figure 1. The sequential path taken for this research effort is indicated in boldface and is described below. The first level of fractionation was applied to the methylene chloride particulate extract. This separation, involving acid/base partitioning and open bed silica gel column chromatography, has been described in detail elsewhere (15). The elution solvents of the silica gel correspond to the designation of the five fractions collected. These five fractions represent the general compound classes of aliphatic hydrocarbons, aromatic hydrocarbons, moderately polar, highly polar and extremely polar neutral compounds.

The second and third levels of fractionation were achieved using normal phase high performance liquid chromatography (HPLC) (Zorbax SiO_2 , 4.6 mm x 25 cm column, Dupont Instrument Co.). The HPLC system was assembled from modular components consisting of an Altex Model 430 gradient programmer with Model 100A pumps (Altex-Beckman Co.) and an LDC Model 1203 UV detector (Laboratory Data Control Co.). A flow rate of 1 mL/min was used. The eluate was monitored for UV absorbance at 254 nm.

The second level of separation, applied to the methylene chloride silica gel fraction (CH_2Cl_2), was achieved by HPLC using a

linear gradient of methanol:methylene chloride (2:98, v:v) in hexane. The gradient was initiated with 8 percent of the methanol:methylene chloride mixture and increased to 35 percent over 30 minutes.

The third level of separation, applied to the third subfraction of the methylene chloride fraction ($\text{CH}_2\text{Cl}_2\text{-C}$), was achieved using a linear gradient of 80 percent methylene chloride in hexane increasing to 100 percent methylene chloride in 30 minutes.

Mass Spectrometry Methods

Mass spectra were recorded using a Finnigan Model 4500 mass spectrometer equipped with an electron impact (EI) chemical ionization (CI) source. For EI analyses, a 70 eV electron beam, emission current of 0.3 mA, and an ion source temperature of 180°C were used. For negative chemical ionization (NCI) analyses, methane was used as the reagent gas at an indicated ion source pressure of 1.0 torr. Methane was ionized by a 150 eV electron beam and the source temperature was maintained at 180°C. Data acquisition was controlled by a Finnigan INCOS Model 2100 data system. A scan rate of 1 second per scan was used for both the EI and NCI analyses. The scan range was 80-450 amu for the EI analyses and 100-450 amu for the NCI analyses.

High resolution gas chromatography (HRGC) for the EI- and NCI-MS analyses was achieved using a Hewlett-Packard Ultra #2 fused silica capillary column (5 percent phenylmethylsilicone bonded phase, 50M x 0.31 mm ID, 0.52 μm film thickness) with methane carrier gas (linear flow velocity of ~75 cm/sec at 40°C). The GC column was interfaced directly to the ion source of the mass spectrometer. On-column injection at 38°C was used. For the EI HRGC/MS analyses, the column temperature was held at 38°C for 2 minutes, programmed ballistically to 100°C in 3 minutes, and programmed 100-320°C at 8°C/minute. For the NCI HRGC/MS analyses, the column temperature was held at 38°C for 0.5 minute, programmed ballistically to 150°C in 3 minutes, and then programmed 150-320°C at 10°C/minute.

Quantification was performed using D9-1-NO₂-pyrene (D9-NP) as the internal standard at a concentration of 2.5 ng/μL. The analyses were performed using on-column injection NCI HRGC/MS with instrumental conditions as described above. The calibration standards covered a 100 fold concentration range.

Chemicals

Reference OH-NO₂-pyrene isomers, 1-NO₂-3-,4-,5-,6-,8-,9-, and 10-OH-pyrene, were obtained from L. M. Ball. The synthesis and characterization of these isomers have been detailed elsewhere (16). Reference 1-NO₂-2-OH-pyrene was obtained from A. Greenberg and 1-NO₂-2-OH-fluoranthene was obtained from B. Zielinska. For the sake of clarity in discussing these isomers, the OH functional group will be listed first, despite conventional nomenclature, since the NO₂ substituent retains position #1 throughout these discussions.

Distribution and Recovery Studies

Reagent spikes in methylene chloride of 3-OH-1-NO₂-pyrene (3-HNP, 12 μg) and mixed 6-OH-1-NO₂- and 8-OH-1-NO₂-pyrene (6/8-HNP, 106 μg) were used for distribution and recovery studies. The first three steps of the fractionation scheme (i.e. acid/base partitioning, silica gel column chromatography, and the second level of fractionation HPLC method) were evaluated separately, each in duplicate. Two of the three fractions resulting from acid/base partitioning, acidic and neutral component fractions, were analyzed. Three of the four silica gel column chromatography fractions, hexane:benzene, methylene chloride, and methanol, were analyzed. For the HPLC method of the second level of fractionation, the HNP were injected and the retention window 31-39 minutes, which encompasses elution of 3-, 6-, 8-HNP, was collected and analyzed. Recovery through the third level of fractionation was estimated by taking the average UV response (n=3) for each compound analyzed by the third level HPLC method, dividing by the

average UV response of each compound analyzed by the second level HPLC method and multiplying by recovery determined above for the second level method. For this study 10 ng of 3-HNP and 30 ng of 6/8-HNP were analyzed each time.

The recovery was also determined for reagent and matrix spikes of 3-HNP (12 µg) and 6/8-HNP (106 µg) in which acid/base partitioning was followed by isolation of the HNP in the acid fraction using the HPLC method of the second level of fractionation. Following injection of the acid fraction, the HPLC retention window 31-39 minutes was collected and analyzed. The methylene chloride extract of the NBS Standard Reference Material 1649 (Washington, D.C. Urban Air Particulate Matter) was used as the matrix material.

Microbial Mutagenicity Methods

The organic extracts and fractions were assayed for mutagenicity toward Salmonella typhimurium strain TA98 with and without exogenous metabolic activation (+S9,-S9) in the Ames plate incorporation assay (17). Minor modifications to this protocol, including the minimal histidine in the base agar and incubation of the plates for 72 hours, have been described in detail elsewhere (18). The plates were counted electronically using an Artek Model 880 automatic colony counter. The studies reported here for TA98 included positive controls both with activation (2-anthramine) and without activation (2-nitrofluorene). Non-linear model slopes were determined from the dose response data (19).

Samples from the first level of fractionation were tested with and without S9 at seven doses with triplicate plates at each dose. Samples from the second level of fractionation were tested with S9 at 3-9 doses with single plates at each dose. Samples from the third level of fractionation were tested with S9 at a single dose in the range of 1-50 µg depending on available sample quantity.

RESULTS AND DISCUSSION

Rationale for Comparing EI and NCI HRGC/MS Analysis Results

To determine whether a complex fraction had been reduced in components to a relatively small number of potential mutagens, the results of EI and NCI HRGC/MS analyses of a fraction were compared. This comparison was used initially to determine whether additional fractionation was required. Further fractionation was not required if (1) individual HRGC/MS chromatographic peaks contained a single component, and (2) the same set of compounds were detected in both EI and NCI analyses. The latter criterion was chosen on the assumption that polar ambient air mutagens, like nitro-PAHs, may be electronegative and present at low concentration levels in the original extract. While EI ionization provides approximately equal ionization efficiency, and hence detection, for all compound classes, NCI greatly favors ionization, and hence detection, of electronegative compounds. If fractionation has substantially reduced the complexity of a subfraction, detection of compounds by both MS techniques may provide reasonable assurance that the significant low level electronegative components of the subfraction have been detected.

After sufficient fractionation, comparisons of EI and NCI mass spectral data were used to select the potentially mutagenic compounds. Ambient air particulate extracts are clearly too complex for sequential fractionation to provide single component subfractions. Our approach was to use sequential fractionation to generate multi-component subfractions and then to rely on the separation powers of HRGC/MS to provide single component identification. The rationale for selecting the potentially mutagenic species in multi-component subfractions was based on the observation that many chemically reactive and mutagenic compounds (e.g., cyclopenta[cd]pyrene, benzo[a]pyrene, 1-nitropyrene) have a higher response under NCI MS conditions than under EI MS conditions. In this work, comparison of EI and NCI HRGC/MS analysis

results has been used to identify as potential mutagens those components which exhibit higher response under the NCI conditions than under EI conditions.

Distribution of Mutagenicity for the First Level of Fractionation

The distribution of direct (-S9) and indirect (+S9) acting mutagenic activity among the seven compound class fractions of this separation are given in Table I. A mutagenicity enrichment factor (MEF), defined here as percent activity divided by percent mass, is also shown for these fractions. The CH₂Cl₂ fraction had a relatively high MEF, 5, and nearly 50 percent of the indirect-acting activity. Analysis of this fraction by EI HRGC/MS showed two major components and ten minor components (phthalate esters, aliphatic aldehydes, aliphatic acid esters, polycyclic aromatic ketones), none of which are known to be mutagenic toward TA98. Due to the high concentration of these compounds, little, if any, other material could be detected.

Distribution of Mutagenicity for Second Level of Fractionation

Normal phase HPLC separation of the CH₂Cl₂ resulted in 86 percent mass recovery. The UV chromatogram and the percentage of recovered mass and of recovered mutagenicity of each subfraction (CH₂Cl₂-A, CH₂Cl₂-B, etc.) are shown in Figure 2. Fraction CH₂Cl₂-C contained nearly 50 percent of the recovered activity and 27 percent of the mass.

Individual peaks in the EI and NCI HRGC/MS chromatograms of CH₂Cl₂-C were relatively broad and contained at least 4-6 distinct molecular ions with overlapping fragment ion spectra. In addition, major components in the NCI analysis were not found in the EI analyses.

Distribution of Mutagenicity for Third
Level of Fractionation

Although reverse phase HPLC could potentially be used to fractionate CH₂Cl₂-C, this approach was rejected since some of the mutagenic material of the fraction was not soluble in the methanol/water solvent system used. Normal phase HPLC conditions provided 85 percent mass recovery and sufficiently good peak shape and resolution to allow separation of CH₂Cl₂-C into 12 subfractions (CH₂Cl₂-C-1, etc.) as shown in Figure 3. The mutagenic activity of subfractions CH₂Cl₂-C-4 and CH₂Cl₂-C-11, 29 and 37 rev/μg, respectively, was higher than the activity of CH₂Cl₂-C. Both fractions were analyzed; results are given here for analyses of CH₂Cl₂-C-4.

Detection of OH-NO₂-PAHs and OH-NO₂-PAKs
in Mutagenic Subfraction CH₂Cl₂-C-4

The complexity of the original CH₂Cl₂ fraction had been reduced such that most HRGC/MS peaks of CH₂Cl₂-C-4 contained a single component. In addition, major components in the NCI HRGC/MS analysis were also detected in the EI HRGC/MS analysis. A retention window from the EI and NCI chromatograms is expanded in Figure 4 for comparative purposes. The elution positions of two phthalate esters, butylbenzylphthalate (BBP) and di-2-ethylhexylphthalate (DEHP), were used as internal standards for relative GC retention correlation between the chromatograms. As seen in Figure 4, component 3 had a slightly different retention value in the two analyses. However, the difference in expected and actual elution time of component 3 in the NCI analysis (Figure 4) was so minor, 9 seconds, as to not affect any conclusions of this study. This difference was apparently due to the fact that although the GC phase was identical for the two analyses, the GC column itself was different.

Four OH-NO₂-PAH/PAKs, including a OH-NO₂-fluorenone isomer, a OH-NO₂-fluoranthene/pyrene isomer, and two OH-NO₂-methylfluorenone isomers, were tentatively identified in the EI and NCI mass spectral analyses of CH₂Cl₂-C-4. The OH-NO₂-fluorenone isomer was detected in both the EI and NCI analyses. The OH-NO₂-fluoranthene/pyrene isomer and two OH-NO₂-methylfluorenone isomers were detected only in the NCI analysis. The identification of component 3 of Figure 4 as OH-NO₂-fluorenone was based on the close agreement of its EI and NCI mass spectral fragmentation patterns with those of a reference OH-NO₂-PAH, 6-OH-1-NO₂-pyrene. The EI and NCI mass spectra of these two compounds are shown in figure 5. Both EI spectra show an intense molecular ion (base peak) and major fragment ions corresponding to the loss of O, NO, CNO₂ and CONO₂ (M-16, M-30, M-58, and M-74 amu, respectively). In addition, the OH-NO₂-fluorenone spectrum contains a fragment ion corresponding to the loss of CO (M-28). The NCI spectra of the OH-NO₂-fluorenone isomer and reference 6-OH-1-NO₂-pyrene show intense molecular ions and fragmentation ions as indicated (M-16, M-30, M-32 and M-46), which are consistent with the structure. The relative intensities of the M-30 and M-32 ions, different for these two compounds, appear to be compound and isomer specific; the ratio of intensity of M-30/M-32 ions covered a 40-fold range (0.1-4.0) in the NCI spectra of seven OH-NO₂-pyrene isomers.

Three other components, OH-NO₂-fluoranthene/pyrene and two OH-NO₂-methyl fluorenones, with NCI fragmentation identical to the OH-NO₂-fluorenone and reference 6-OH-1-NO₂-pyrene were tentatively identified. The high mutagenic activity of NO₂-fluoranthenes and NO₂-pyrenes indicated that confirmation of the identification of a OH-NO₂-fluoranthene/pyrene isomer should be pursued.

Distribution and Recovery of OH-NO₂-Pyrene Isomers Through Fractionation

Analyses of reference OH-1-NO₂-pyrene (HNP) isomers (3-, 4-, 5-, 6-, 8-, 9- and 10-OH-1-NO₂) by the HPLC methods used in the second and third levels of fractionation demonstrated that, on the basis of

solubility and polarity, a OH-NO₂-fluoranthene/pyrene isomer might elute in the HPLC windows designated as CH₂Cl₂-C and CH₂Cl₂-C-4. The shaded regions of the chromatograms shown in Figure 6 correspond to the retention windows of CH₂Cl₂-C (28-35 min), and CH₂Cl₂-C-4 (9-14.5 min), respectively. Four of the seven HNP isomers eluted in the CH₂Cl₂-C window and three of the seven HNP isomers eluted in the CH₂Cl₂-C-4 window.

The results of the distribution and recovery studies for 3- and 6/8-HNP in each of the steps used in isolation of CH₂Cl₂-C-4 demonstrated that a OH-NO₂-fluoranthene/pyrene isomer might be detected in a polar neutral fraction. A large percentage of the HNP (>85 percent) partitioned into the acid fraction, but a quantifiable amount remained in the neutral fraction. On 5 percent water-deactivated silica gel, these HNP eluted primarily in the methylene chloride fraction. Recovery from the silica gel HPLC column in which the mobile phase was modified with methanol was significantly better, as expected, than recovery from that column without methanol in the mobile phase. (Note: The retention windows used in these HPLC analyses correspond to elution of 3, 6 and 8-HNP, rather than to the retention windows of CH₂Cl₂-C and CH₂Cl₂-C-4.) Results of these studies are compiled in Section A of Table II.

The distribution and recovery studies showed that, although a OH-NO₂-fluoranthene/pyrene isomer could be found in a polar neutral fraction (methylene chloride silica gel fraction), a larger quantity of the compound should be detected in the organic acid fraction of the extract. Section B of Table II shows recovery results for reagent and matrix spikes of 3-HNP and 6/8-HNP in which acid/base partitioning was followed by isolation of the HNP in the organic acid fraction using the HPLC method of the second level of fractionation. By this procedure, recovery of the HNP isomers was slightly higher than 70 percent for the reagent spikes and averaged 50 percent for the matrix spikes. These results demonstrated that separation of an organic acid fraction on a silica gel HPLC column, while possibly not ideal for recovery of all

fraction components, might be used to recover a quantifiable amount of a OH-NO₂-fluoranthene/pyrene isomer.

Quantification of HNP by NCI HRGC/MS is demonstrated in Figure 7 by the calibration curve with dynamic range of 100. The calibration curve is higher than first order in the independent variable, possibly due to decomposition mechanisms higher than first order. The molar response of 6/8-HNP relative to Dg-NP for these data, ranging from 0.74 for 50 ng of HNP to 0.10 for 0.5 ng of HNP, is higher than the molar response of 0.05 reported elsewhere (20). The relatively high response here is most likely due to our use of the cold on-column injection technique, as opposed to heated splitless injection used by the latter. Good chromatography was observed for these compounds without derivatization of the OH substituent group. Peak width at half height was 3-4 seconds and peak tailing was minimal.

Evidence for the OH-NO₂-Fluoranthene Identification

The organic acid fraction (Acid) of the ambient air particulate extract was separated using the HPLC method of the second level of fractionation. The retention window 28-35 minutes, that being the elution of retention window CH₂Cl₂-C, was collected, designated Acid-C, and analyzed by NCI HRGC/MS. The OH-NO₂-fluoranthene/pyrene isomer tentatively identified in CH₂Cl₂-C-4 was detected in this Acid-C fraction. The NCI mass spectra of this compound in both Acid and CH₂Cl₂ fractions correspond extremely well, as shown in Figure 8, and their HRGC/MS relative retentions were identical, 0.89 versus DEHP. An EI mass spectrum of this compound has not yet been obtained.

In lieu of an authentic standard at this time, evidence is presented here to support tentative identification of this compound as a OH-NO₂-fluoranthene isomer. First, presence in the organic acid fraction requires a molecule with a functional group such as -OH. Second, the NCI mass spectrum of this compound contains not only the (M-O)⁻, (M-NO)⁻, (M-NO₂)⁻ ions, but also an ion at m/z 201 (1 percent, S/N = 10) which would correspond to (M-O-NO₂)⁻. Third, all OH-1-NO₂-pyrene

isomers analyzed have retention values relative to Dg-1NP which are significantly different (see Table III). The 7-HNP isomer would not be expected to elute earlier than 2-HNP. Although NO₂-fluoranthene/pyrenone compounds are isomeric, they would not be expected in an acid fraction. Such compounds have been tentatively identified by a characteristic M-CO loss in NCI mass spectra in hexane:benzene silica gel fractions of other particulate extracts (diesel engine) with retention values relative to NP of 1.032 and 1.040 (8).

The early GC elution of this tentatively identified OH-NO₂-fluoranthene relative to the NO₂-fluoranthene isomers (see Table III) is unexpected. However, the retention order of other OH-NO₂-PAHs (see Table IV), tentatively identified in this same Acid-C fraction on the basis of their EI HRGC/MS spectra, is unexpected. For example, a OH-NO₂-acenaphthene (MW 215) elutes earlier than a OH-NO₂-naphthalene (MW 189). Retention order may not be predicted for OH-NO₂-PAHs as readily as for PAHs (21) and NO₂-PAHs (22). For the pyrene nucleus, adjacent carbon substitution (2-OH-1-NO₂) produces the earliest eluting OH-NO₂ isomer. Substitution of fluoranthene in the bay region, 10-OH-1-NO₂, may produce a compound which through intramolecular hydrogen bonding is substantially less polar than the NO₂-PAH parent and nearly as compact in size.

Quantification of this OH-NO₂-fluoranthene isomer in the organic acid and methylene chloride extract fractions is shown in Table V. The mass distribution of this compound between these two fractions was found to be 99.7 and 0.3 percent, respectively. This distribution compares well with the distribution between acid and neutral fractions, 99.8 and 0.2 percent, respectively, determined for the reference HNP isomers in the recovery study (Table II, Section A). Calculated values for the level of this OH-NO₂-fluoranthene in the particulate extract, the MAVS Stage III particles, and in the ambient air of the site are included in Table V. For comparison, the level of 2-NO₂-fluoranthene and 1-NO₂-pyrene in this ambient air sample are shown in Table V, also.

Significance of Detection of OH-NO₂-PAHs

The presence of OH-NO₂-PAHs in particulate matter has been suggested by Löfroth, Nielsen and Schuetzle to account for mutagenicity in extract fractions more polar than those containing NO₂-PAHs (23,24,25). Similar mechanisms have been proposed by which atmospheric hydroxylation of PAHs followed by nitration readily yields both NO₂-PAHs and OH-NO₂-PAHs (24,25,26). The detection of a OH-NO₂-fluoranthene isomer in an ambient air particulate extract should not be unexpected since 2-fluoranthene has been identified and quantified in this particulate extract at 6.4 µg/g extract mass (27).

Three OH-NO₂-pyrene isomers were tentatively identified by Schuetzle (25) in diesel particle emission extracts using a tandem mass spectrometric (MS-MS) technique. Schuetzle estimated the concentration of OH-NO₂-pyrenes to be in the range of 10-100 µg/g extract. Based on the mutagenicity of a mixture of 3-, 6- and 8-OH-1-NO₂-pyrenes, the contribution to the mutagenicity of one diesel extract was estimated to be as high as 5 percent (20).

The importance of OH-NO₂-pyrenes became apparent when they were first identified as mutagenic mammalian metabolites of 1-NO₂-pyrene (16). The mutagenicity of the OH-NO₂-pyrene isomers tested (3-,4-,5-, 6-,8-,9- and 10-OH-1-NO₂) ranged from 0.06 to 8 times the activity of 1-NO₂-pyrene (28). The OH-NO₂-pyrene isomers are generally more mutagenic in the absence of activation and appear to be metabolized in vivo to the corresponding ring-hydroxylated-N-acetyl-aminopyrenes, which are also mutagenic but require activation.

The higher mutagenic activity of the NO₂-fluoranthene (NF) isomers [8 NF, 42,000 rev/µg; 3 NF, 32,000; 2 NF, 2000; 1 NF, 1900 (29,30)] versus 1-NO₂-pyrene [1000 rev/µg (7)] suggests that OH-NO₂-fluoranthenes may be more mutagenic than OH-NO₂-pyrenes. The identification of both a NO₂-fluoranthene and a OH-NO₂-fluoranthene isomer in an ambient air particulate extract underscores the importance of elucidating the potential health effects of these compounds.

CONCLUSIONS

This study has demonstrated that bioassay-directed fractionation and characterization is a highly effective method for detecting and identifying heretofore unknown and potentially mutagenic constituents of an extremely complex ambient air particulate extract. Three sequential fractionation techniques of increasing chromatographic resolution produced a mutagenic subfraction that was reduced in chemical complexity such that individual HRGC/MS peaks were single components. Several OH-NO₂-PAHs/PAKs were tentatively identified in this subfraction. The identifications were based on NCI and EI mass spectra of these compounds with reference HNP and a study of distribution and recovery of HNP through the fractionation steps used. The HRGC/MS retention values for HNP isomers indicated that one of the OH-NO₂-PAHs detected in this ambient air sample may be a OH-NO₂-fluoranthene isomer. This OH-NO₂-fluoranthene isomer has been quantified in both the organic acid and moderately polar neutral silica gel fractions. Based on the levels of NO₂-PAHs in this ambient air particulate extract, the level of this tentatively identified OH-NO₂-fluoranthene is sufficiently high to suggest that if mutagenic, this compound may help account for mutagenicity in polar fractions.

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TABLE I. DISTRIBUTION OF MASS AND MUTAGENIC ACTIVITY FOR FIRST LEVEL OF FRACTIONATION

Fraction	Mass, %(b)	Mutagenic Activity, (a)			
		-S9(c)		+S9(d)	
		%(e)	MEF(f)	%	MEF
Organic Acids	7	38	5.4	21	3.0
Organic Bases	1	1	1.0	1	1.0
Hexane	21	0	0.0	0	0.0
Hexane: Benzene	14	8	0.6	12	0.9
Methylene Chloride	9	23	2.6	45	5.0
Methanol	34	29	0.9	21	0.6
Acidic Methanol	14	1	<0.1	<1	<0.1

- (a) Mutagenic activity toward Salmonella typhimurium TA98.
(b) Mass recovery, 94% (15).
(c) Direct acting mutagenic activity recovery, 84% (15).
(d) Indirect acting mutagenic activity recovery, 88% (15).
(e) Percent distribution of recovered activity
(f) Mutagenicity enrichment factor: % activity divided by % mass.

TABLE II. DISTRIBUTION AND RECOVERY OF OH-NO₂-PYRENE ISOMERS
IN FRACTIONATION STEPS

	Percent Recovery	
	3-HNP	6/8-HNP
<u>A. Separately Performed Steps-Reagent Spikes</u>		
<u>Fractions Analyzed</u>		
<u>Acid/Base Partitioning(a,b)</u>		
Acid Fraction	102	86
Neutral Fraction	0.2	0.2
Total Recovery	102	86
<u>Silica Gel Column Chromatography(a,c)</u>		
Hexane:Benzene	ND(d)	ND
Methylene Chloride	12	53
Methanol	7	5
Total Recovery	19	58
<u>Silica Gel HPLC (Methanol:Methylene Chloride in Hexane Gradient)(a,e)</u>		
Retention Window 31-39 min	73	78
<u>Silica Gel HPLC (Hexane and Methylene Chloride Gradient)(f)</u>		
Retention Window 9-25 min	25	52
<u>B. Combined Steps</u>		
<u>Acid/Base Partitioning and Silica Gel HPLC</u>		
Reagent Spike - Retention Window 31-39 min(g)	77	71
Matrix Spike - Retention Window 31-39 min(h)	60	45

- (a) Duplicate analyses.
- (b) Spike solution diluted to 50 mL with MeCl₂; partitioned 3 times with 40 mL of NaOH and H₂SO₄; aqueous phase back extracted 3 times with 40 mL MeCl₂; 3-HNP analyses $\pm 10\%$; 6/8-HNP analyses $\pm 10\%$.
- (c) Spike solution solvent exchanged with hexane and placed at head of 10 g silica gel column; column eluted with 80 mL hexane and 140 mL each of hexane:benzene, methylene chloride and methanol; 2 mL of each elution solvent, used to rinse reagent spike vessel, was used as initial portion of eluent; 3-HNP analyses $\pm 5\%$; 6/8-HNP analyses $\pm 10\%$.
- (d) None detected.
- (e) Reagent spike concentrated to 100 μ L; multiple analyses by HPLC method with pooled collection of retention window listed; analyses $\pm 5\%$.
- (f) Recovery estimated as ratio of HPLC UV response for triplicate analyses of standards using each HPLC gradient multiplied by recovery of preceeding step.
- (g) Acid/base partitioning performed as in footnote (b) and acid fraction separated by HPLC as in footnote (e).
- (h) Spike solution added to 50 mg of methylene chloride extract of NBS Standard Reference Material 1649 (Washington, D.C. Urban Air Particulate Matter); sample separated as in footnote (g).

TABLE III. RELATIVE RETENTION VALUES FOR SELECTED NO₂-FLUORANTHENE/
NO₂-PYRENE ISOMERS AND OH-NO₂-FLUORANTHENE/OH-NO₂-
PYRENE ISOMERS

Compound	Retention Relative to D9-1NP
Tentatively identified OH-NO ₂ -fluoranthene	0.865
1-NO ₂ -fluoranthene	0.944
7-NO ₂ -fluoranthene	0.951
2-NO ₂ -fluoranthene	0.966
3-NO ₂ -fluoranthene	0.971
2-OH-1-NO ₂ -fluoranthene	0.991
8-NO ₂ -fluoranthene	0.998
1-NO ₂ -pyrene	1.002
3-OH-2-NO ₂ -fluoranthene(a)	1.008
2-OH-1-NO ₂ -pyrene	1.041
10-OH-1-NO ₂ -pyrene	1.112
9-OH-1-NO ₂ -pyrene	1.203
4-OH-1-NO ₂ -pyrene	1.203
3-OH-1-NO ₂ -pyrene	1.217
6-OH-1-NO ₂ -pyrene	1.232
8-OH-1-NO ₂ -pyrene	1.232
5-OH-1-NO ₂ -pyrene	1.232

(a) Major product formed from direct nitration of 3-OH-fluoranthene and NaOH fusion of 2-NO₂-fluoranthene - private communication, L. M. Ball.

TABLE IV. RETENTION VALUES AND EI MS ION INTENSITIES OF OTHER TENTATIVELY IDENTIFIED OH-NO₂-PAH IN ACID-C FRACTION

Tentative Identification	Retention Relative to D9-NP	Mass (Percent Relative Intensity)							
		M	M-O	M-OH	M-NO	M-NO ₂	M-CNO ₂	M-OH-NO ₂	M-CONO ₂
OH-NO ₂ -benzene	0.395	139(100)	123(19)	ND(a)	109(36)	93(23)	81(20)	--(b)	--
OH-NO ₂ -methyl benzene	0.425	153(41)	137(11)	136(100)	123(4)	107(10)	95(10)	90(21)	--
	0.438	153(100)	137(15)	ND	123(29)	107(14)	95(6)	90(<1)	--
OH-NO ₂ -C ₂ alkyl benzene	0.460	167(100)	151(16)	ND	137(25)	121(13)	109(5)	104(2)	93(10)
	0.469	167(80)	151(14)	150(100)	137(3)	121(10)	ND	104(8)	93(8)
	0.483	167(100)	151(18)	150(50)	137(16)	121(14)	109(3)	104(14)	93(6)
OH-NO ₂ -naphthalene	0.705	189(100)	173(14)	172(5)	159(23)	143(26)	131(23)	126(9)	115(89)
OH-NO ₂ -methyl naphthalene	0.693	203(100)	187(8)	186(24)	173(27)	157(20)	145(14)	ND	129(31)
OH-NO ₂ -acenaphthene	0.685	215(100)	199(8)	198(1)	185(14)	169(5)	157(9)	152(3)	141(36)
OH-NO ₂ -methyl acenaphthene	0.810	229(100)	213(7)	212(ND)	199(40)	183(24)	171(14)	166(5)	155(35)
	0.921	229(100)	213(24)	212(ND)	199(36)	183(18)	171(15)	166(ND)	155(56)

(a) Not Detected.

(b) Instrument not scanned low enough to detect this ion.

TABLE V. CONCENTRATION OF OH-NO₂-PAH AND NO₂-PAHS
IN AMBIENT AIR PARTICULATE SAMPLE

Component	Concentration in Component Material		
	OH-NO ₂ - Fluoranthene	2-NO ₂ - Fluoranthene(a)	1-NO ₂ - Pyrene
Organic Acid Fraction	70 µg/g	NA(b)	NA
Hexane:Benzene Fraction	NA	50 µg/g	0 µg/g
Methylene Chloride Fraction	0.1 µg/g	NA	NA
Particulate Extract(c)	4.8 µg/g	6.4 µg/g	2.6 µg/g
Stage III Particles(d)	0.19 µg/g	0.25 µg/g	0.10 µg/g
Ambient Air(e)	0.008 ng/g	0.01 ng/m ³	0.004 ng/m ³

(a) Recent analyses with reference 2-NF and 3-NF have shown that 2-NF incorrectly identified as 3-NF in Wise, et al. (27).

(b) Not applicable.

(c) CH₂Cl₂ extract of MAVS Stage III particles.

(d) MAVS Stage III particles, <1.7 µm.

(e) Respirable particles, <1.7 µm.

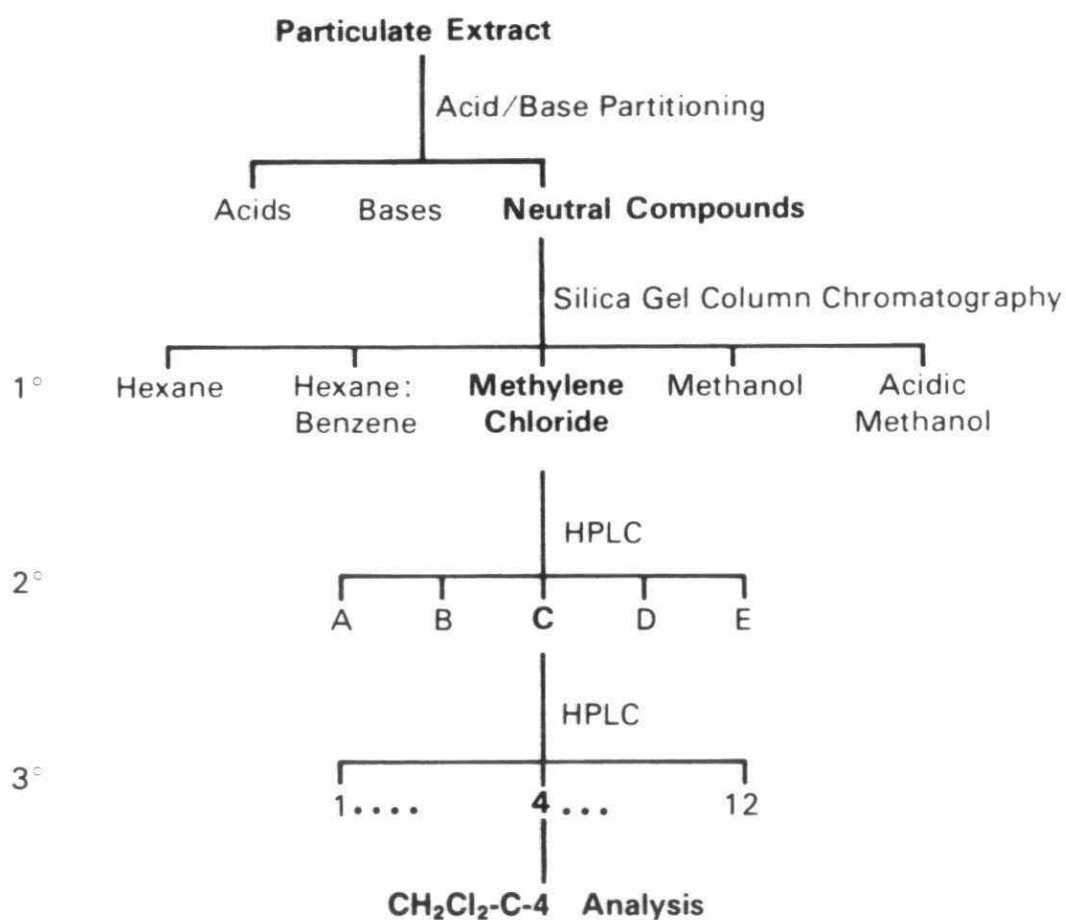


FIGURE 1. OVERVIEW OF FRACTIONATION SCHEME. BOLD FACE TYPE INDICATES PATHWAY LEADING TO ANALYSIS OF MUTAGENIC SUBFRACTION CH₂Cl₂-C-4.

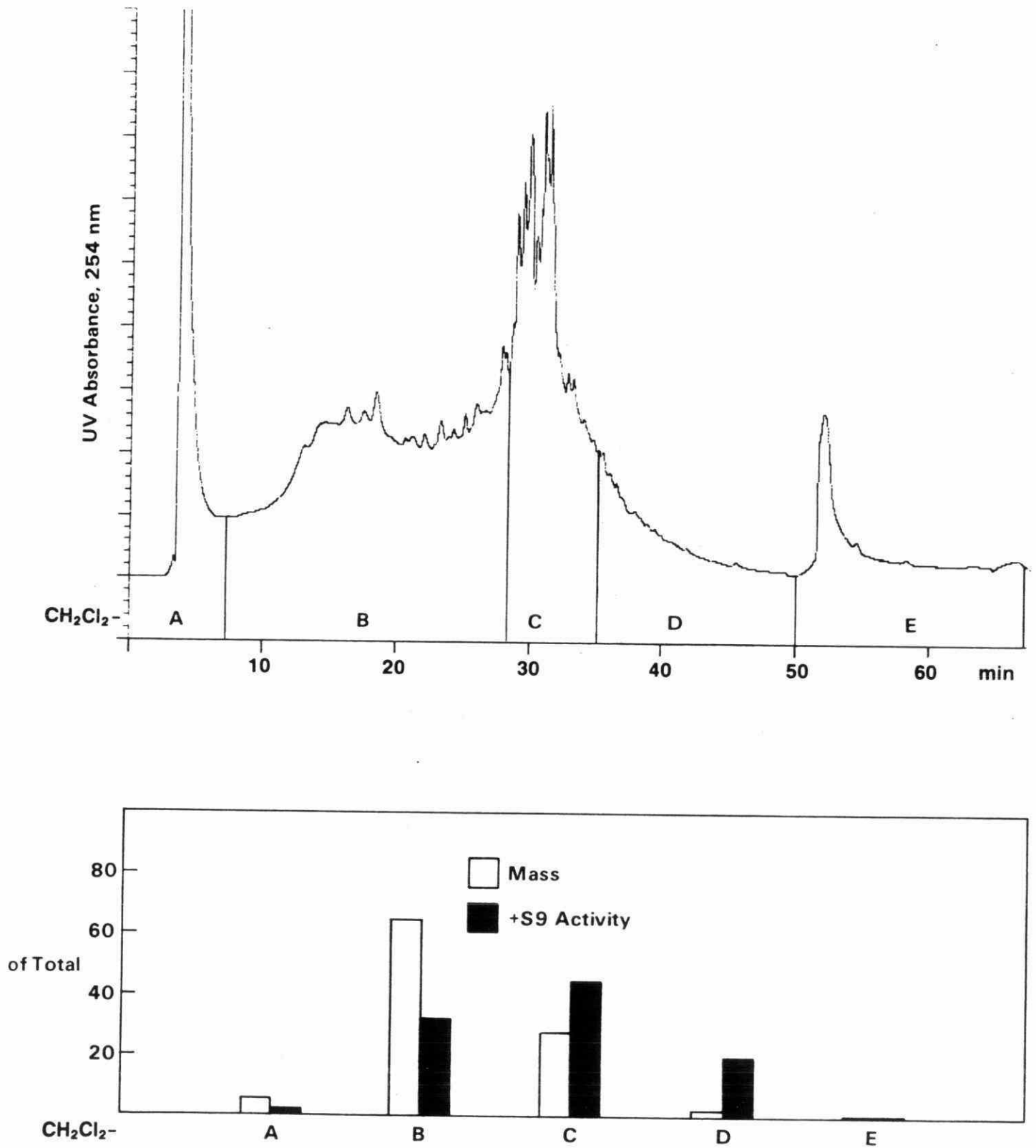


FIGURE 2. UV CHROMATOGRAM FOR HPLC SEPARATION OF CH₂Cl₂ FRACTION. PERCENTAGE DISTRIBUTION OF RECOVERED MASS AND ACTIVITY (TA 98, +S9) OF FIVE SUBFRACTIONS INCLUDED.

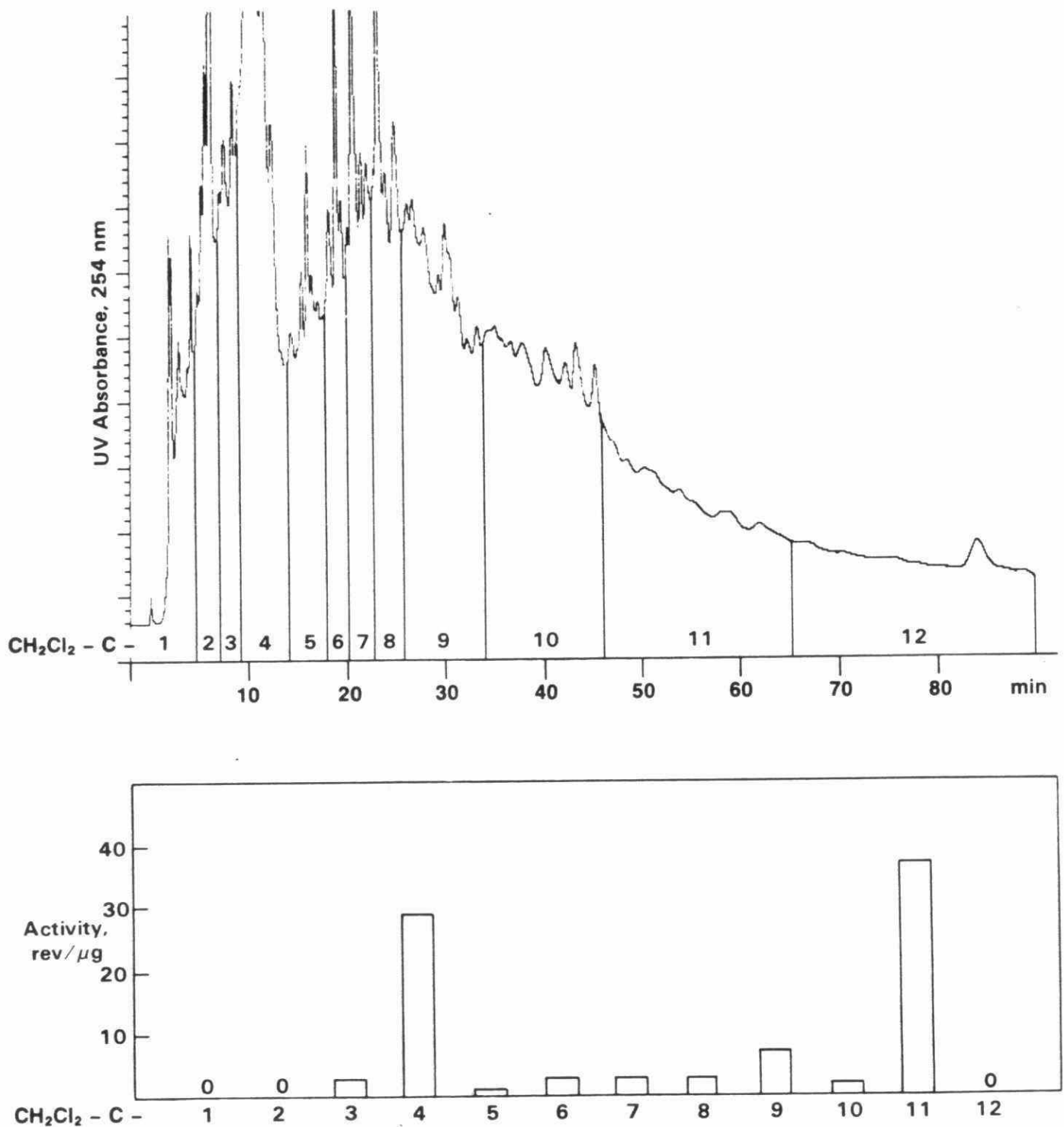


FIGURE 3. UV CHROMATOGRAM FOR HPLC SEPARATION OF CH₂Cl₂-C FRACTION. MUTAGENIC ACTIVITY (TA 98, +S9) OF TWELVE SUBFRACTIONS INCLUDED.

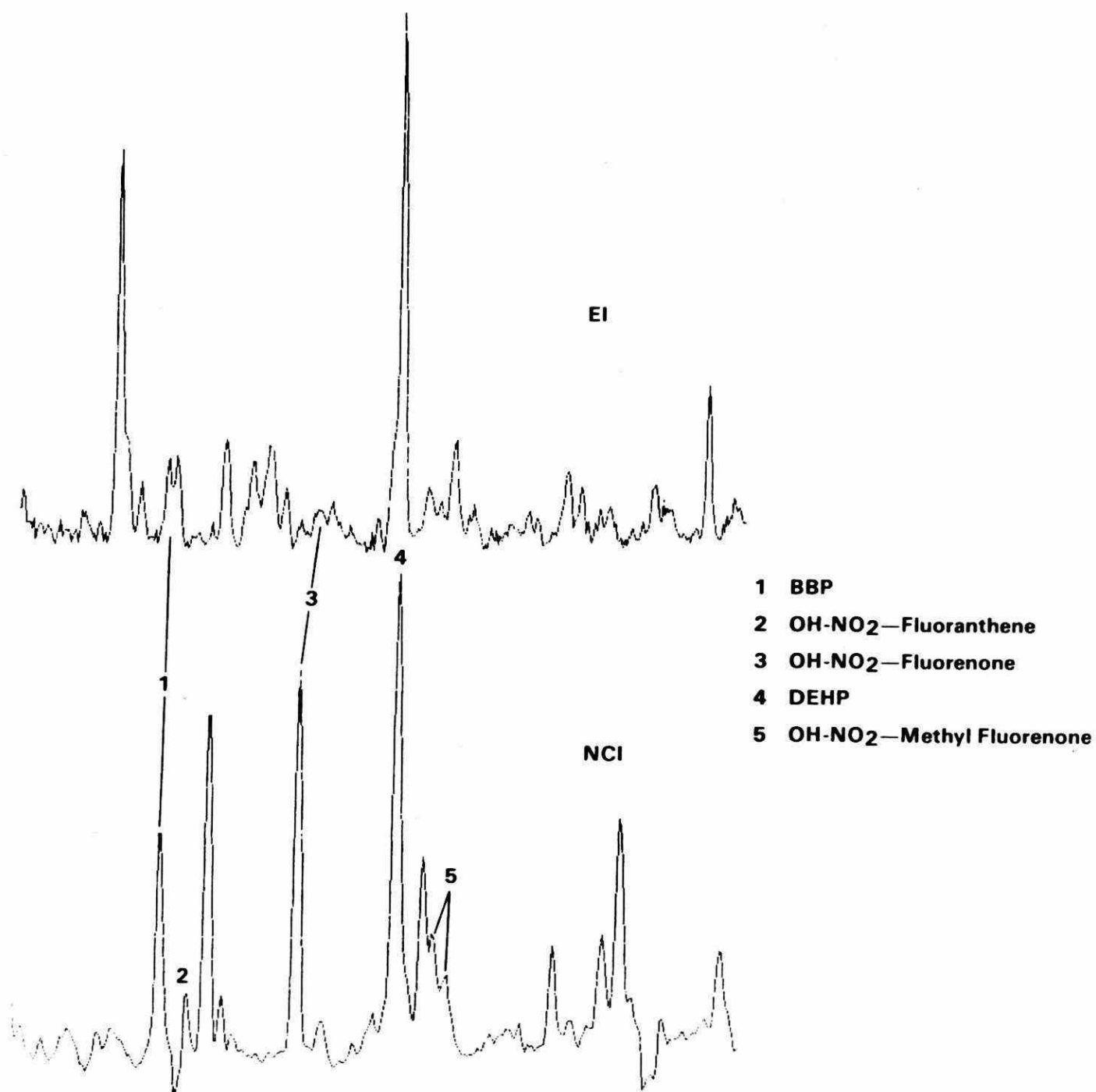


FIGURE 4. SELECTED RETENTION WINDOWS FROM EI AND NCI HRGC/MS ANALYSES OF CH₂Cl₂-C-4. BBP (BUTYLBENZYLPHTHALATE) AND DEHP (DI-2-ETHYLHEXYLPHTHALATE) USED TO ALIGN THE TWO CHROMATOGRAMS.

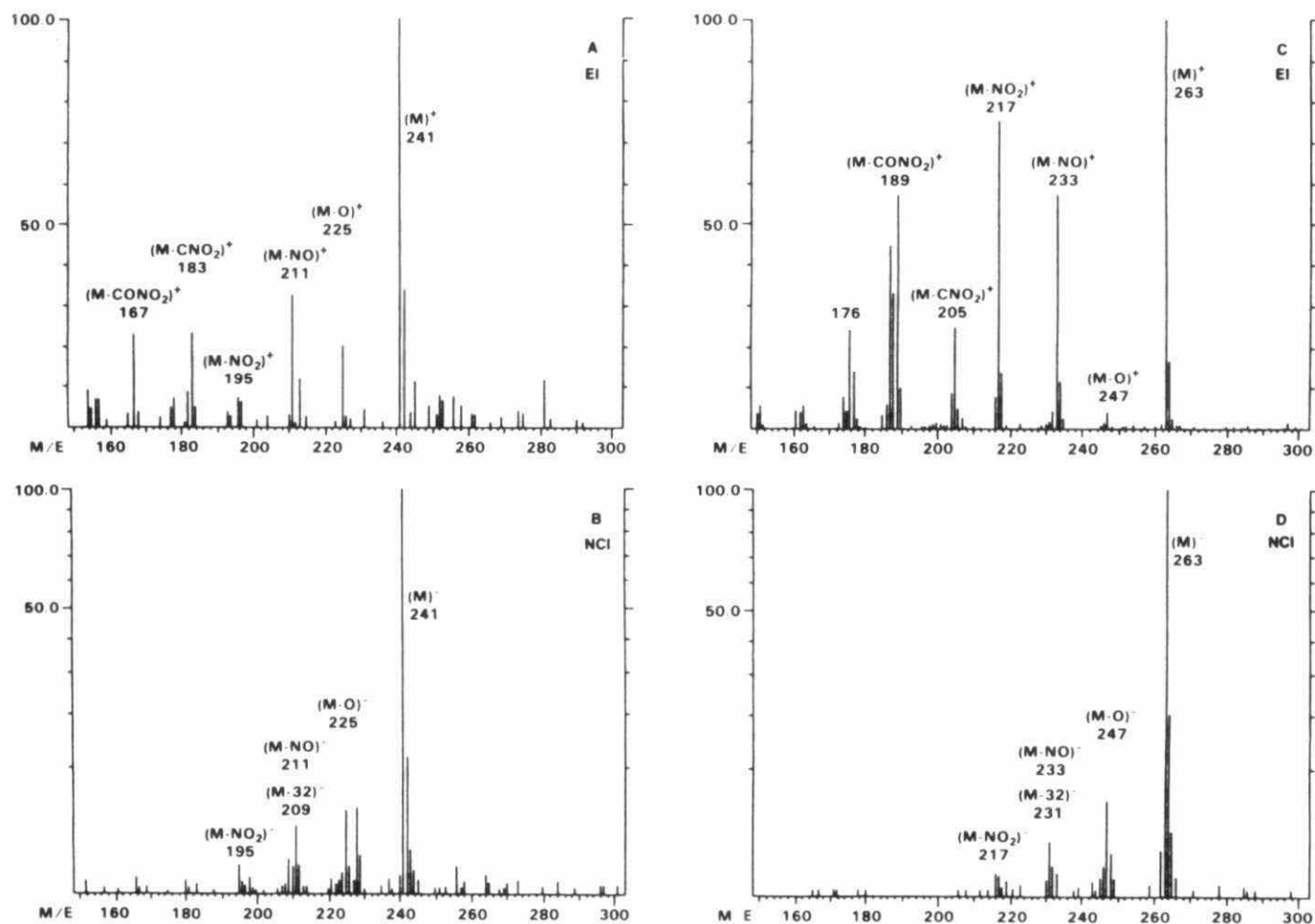


FIGURE 5. MASS SPECTRA OF OH-NO₂-PAHs. OH-NO₂-FLUORENONE IN CH₂Cl₂-C-4: A - EI SPECTRUM, B - NCI SPECTRUM. REFERENCE COMPOUND 6-OH-1-NO₂-PYRENE: C - EI SPECTRUM, D - NCI SPECTRUM. EI SPECTRA - LINEAR INTENSITY SCALE. NCI SPECTRA - SQUARE ROOT INTENSITY SCALE USED TO EXPAND THE DISPLAY OF LOW INTENSITY FRAGMENT IONS.

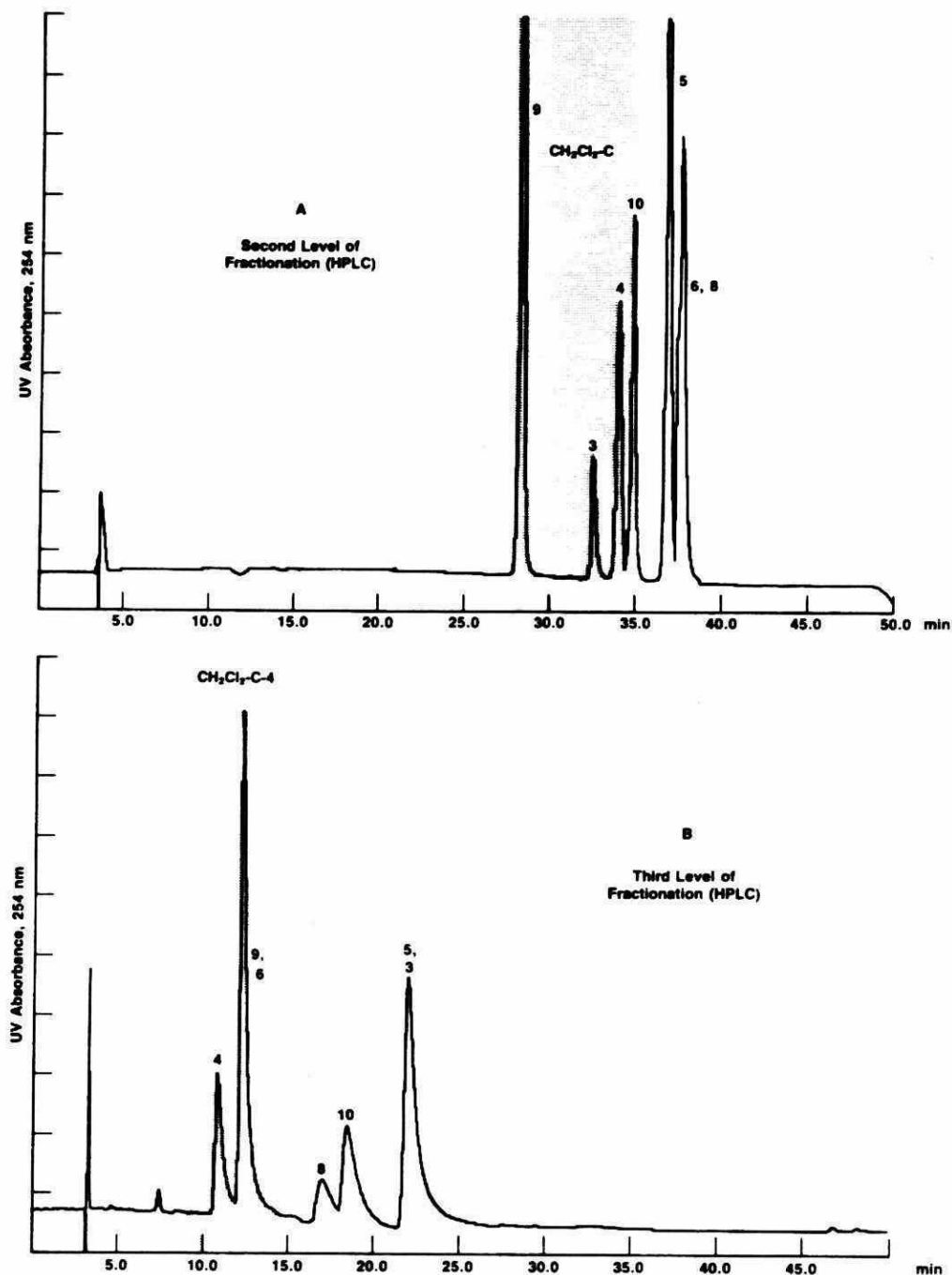


FIGURE 6. HPLC UV CHROMATOGRAMS OF HNP ISOMERS. A - HPLC GRADIENT OF METHANOL/METHYLENE CHLORIDE (2:98, v:v) IN HEXANE, USED AS SECOND LEVEL OF FRACTIONATION FOR SEPARATION OF PARTICULATE EXTRACT FRACTION CH_2Cl_2 . SHADED REGION CORRESPONDS TO RETENTION VOLUME OF $\text{CH}_2\text{Cl}_2\text{-C}$. B - HPLC GRADIENT OF METHYLENE CHLORIDE IN HEXANE, USED AS THIRD LEVEL OF FRACTIONATION FOR SEPARATION OF PARTICULATE EXTRACT FRACTION $\text{CH}_2\text{Cl}_2\text{-C}$. SHADED REGION CORRESPONDS TO RETENTION VOLUME OF $\text{CH}_2\text{Cl}_2\text{-C-4}$. NUMERALS IN FIGURE INDICATE THE OH SUBSTITUENT POSITION OF OH-1- NO_2 -PYRENE ISOMERS ANALYZED.

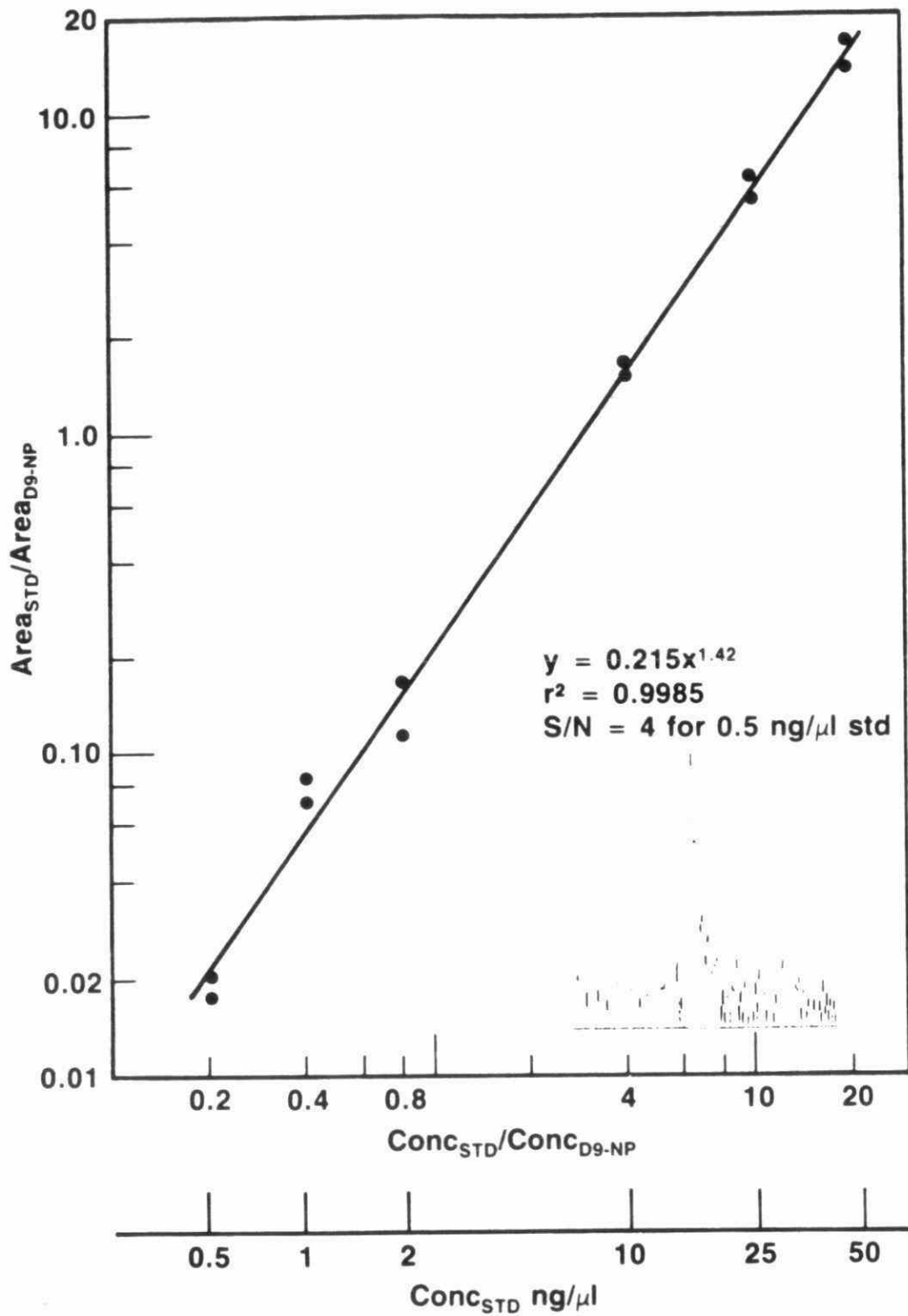


FIGURE 7. REPRESENTATIVE CALIBRATION CURVE FOR NCI HRGC/MS QUANTIFICATION OF 6/8-HNP.

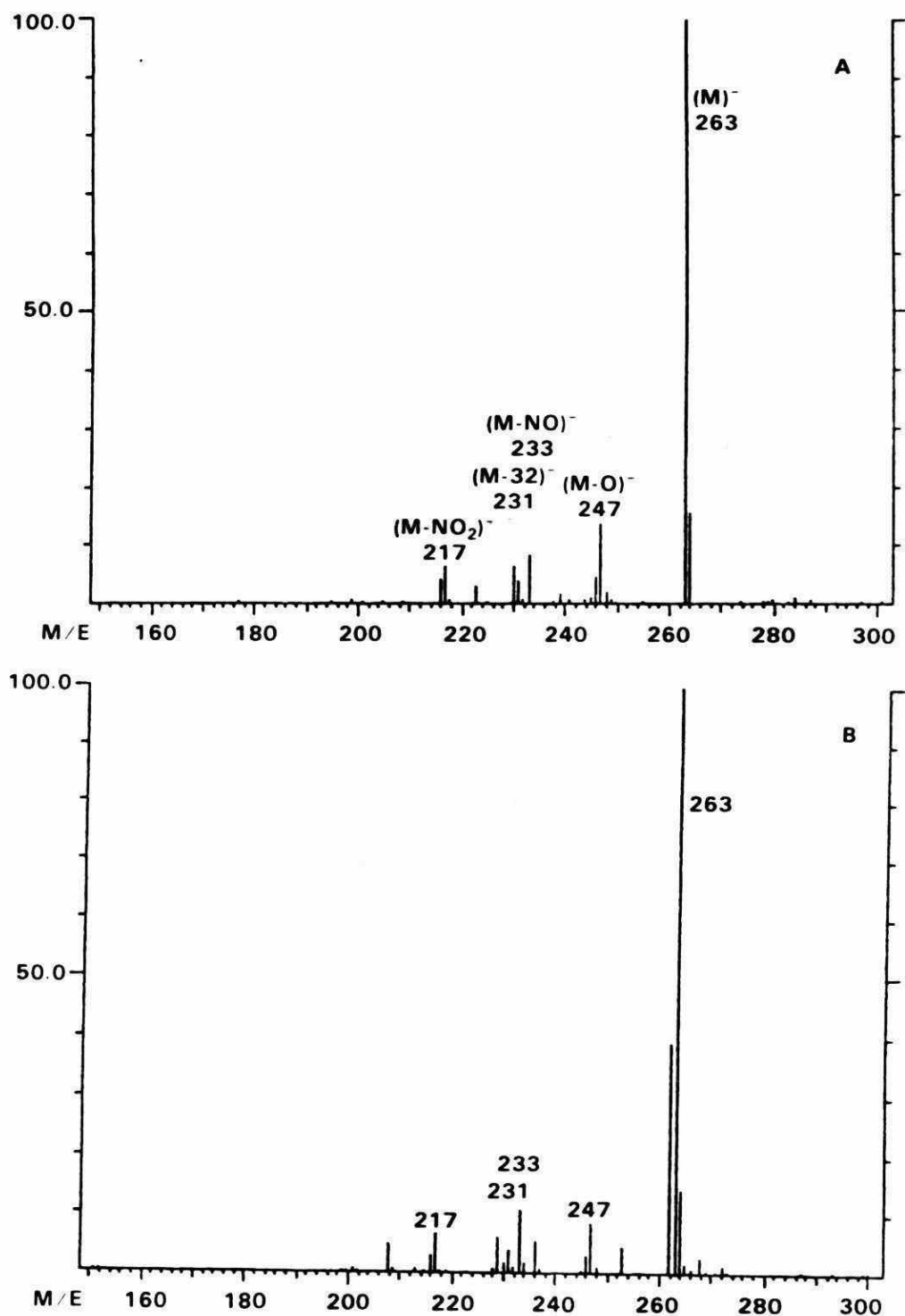


FIGURE 8. NCI MASS SPECTRA OF OH-NO₂-FLUORANTHENE ISOMER IN AMBIENT AIR PARTICULATE EXTRACT. A - SPECTRUM OF COMPOUND IN CH₂Cl₂-C-4 FRACTION. B - SPECTRUM OF COMPOUND IN ACID - C FRACTION.

**SOLID SAMPLE INTRODUCTION
INTO THE INDUCTIVELY COUPLED
PLASMA.**

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SOLID SAMPLE INTRODUCTION INTO THE INDUCTIVELY COUPLED PLASMA.

1. LIQUID SAMPLE ANALYSIS

1.1 ATOMIC EMISSION WITH THE INDUCTIVELY COUPLED PLASMA

1.1.1 ABSTRACT:

A wire loop was used with the direct sample insertion device (DSID) for atomic emission spectrometry with the inductively coupled plasma (ICP). Detection limits are improved by approximately a factor of 10 to 100. Spectral overlaps can in some cases be eliminated by the temporal resolution provided by the wire loop system. Certain matrix effects which disturb conventional sample introduction systems are eliminated or minimized by this system.

1.1.2. MAIN BODY.

Before embarking on the direct analysis of solid samples using the Direct Sample Insertion Device (DSID) for the Inductively Coupled Plasma (ICP) it was deemed useful to gain knowledge of the behavior of the DSID with desolvated liquid samples. In attempting to determine the physical temperature of a graphite electrode placed in the plasma by the melting points of metals, it was found that tungsten wire could be inserted into the plasma with no effect other than the embrittlement of the wire. A new direct sample insertion device was designed and constructed to permit rapid placement of a tungsten wire loop into the plasma. This device was pneumatically driven to provide near instantaneous entry of the wire into the plasma as was found necessary by the short vaporization times of the sample elements. This system has since been replaced with a more versatile stepper motor driven system which will be discussed in section 1.3.

Liquid samples (10ul) placed on the wire are desolvated by placing them at roughly 20 to 30 mm below the top of the load coil with the plasma ignited. The heat of the plasma and the flow of gases around the wire ensured rapid (10 to 30 s) drying of the sample on the wire. This drying step is easily monitored since three phenomena are observed during this time: 1) the plasma constricts itself due to the presence of di- and poly- atomic molecules 2) the reflected power increases slightly 3) the signal intensity rises as the plasma is constricted into the viewing zone. Once dry, the wire is inserted into the plasma with the transient emission signal occurring 50 to 400 ms after insertion and having a half width of approximately 100 ms. Under fixed conditions, the standard deviation on the time occurrence of the analyte peak emission is about 3 ms. The extremely high precision of this occurrence may allow spectrally overlapping lines to be resolved in the time domain. We have observed temporal resolution in at least one case.

With the wire loop direct sample insertion device, the detection limits listed in Table I have been obtained. Table I compares the detection limits obtained with the wire loop DSID to those obtained by Winge et al. (*Appl. Spectrosc.*, 1979, 206-219) using nebulization and which are often used as a comparative measure. The wire loop DSID is providing detection limit improvements of a factor of 10 to 100. The relative standard deviation of the signal well above the detection limit is occasionally as good as 1% but is typically 5 to 10% for manual sample delivery. An automated sample deposition device has been developed to operate with the stepper motor driven DSID described in section 1.3. With the automated sample deposition, the relative standard deviation is consistently 1 to 2 % over periods as long as 3 hours.

Table I. Wire Loop DSID Detection Limits.

Element	Wavelength nm	Detection Limit		
		Conc. ng/ml	Absolute pg	Fassel ng/ml
Copper	324.754	0.2	2	5.4
Zinc (a)	213.856	0.07	0.7	1.8
Cadmium (a)	228.802	0.3	3	2.7
Silver	328.068	0.04	0.4	7
Lead (a)	220.351	3	30	42
Magnesium	280.269	0.007	0.07	0.30

(a) Tantalum wire required due to insufficient temporal resolution from Tungsten spectral overlap.

Difficulties have arisen in the use of Tungsten as a sample support. A certain amount of Tungsten oxidizes during the drying of the liquid sample and, because of the large number of Tungsten lines, spectral overlaps are common. The temporal resolution of the overlaps is not sufficient in many cases (Pb,Cd,Zn) and in others, the processing of the data is rendered more difficult. Experiments with Tantalum show that it would be preferable to Tungsten in many aspects. Tantalum has fewer lines than Tungsten and is less reactive and so should and does provides fewer spectral interferences. Equally important, Tantalum oxide is the least volatile of the oxides consequently ensuring adequate temporal resolution of spectral overlaps. Unfortunately, the lifetime of tantalum sample supports is significantly shorter than that of tungsten sample supports. This requires frequent replacement of the tantalum sample supports. Experiments have shown that, given the present method of manufacturing the sample supports and mounting them on the DSID, the change in signal resulting from the replacement of sample supports is of the order of 5%. It is expected that improved sample support manufacturing and mounting can possibly eliminate any change in response.

The weight, more specifically the total heat capacity, of the sample support has been found to contribute significantly to the performance of the DSID technique which exhibits DC arc type response. With a tungsten wire of twice the mass, the peak occurred approximately 1.5 times later and had a half width 1.5 times longer. The S/N level was one half that of the thinner wire. The geometric configuration of the support also plays an important role, it appears that the larger the exposed area, the more rapid the heating and evolution of the analyte. The different elements volatilize at different times, the order of which is quite similar to that found in DC arc spectrometry. This differential volatilization is beneficial in that spectral overlaps are temporally resolved in many cases.

In the presence of high salt concentrations (1000 ppm Ca) the peak heights and shapes are altered but the peak areas remain essentially the same as for the pure aqueous standards. This indicates a relative freedom from matrix effects, since the total emission signal is directly related to the concentration. Our later work with inductively coupled plasma (ICP) mass spectrometry (MS) confirms these findings. This will be discussed in section 1.2.

1.2 WIRE LOOP DIRECT SAMPLE INSERTION FOR ICP SOURCE MASS SPECTROMETRY

1.2.1 ABSTRACT.

The wire loop technique was used to introduce aqueous samples into the ICP for mass spectrometry. A new device was constructed and installed at the Rexdale MOE facility. Mass Spectrometry detection limits were improved by a factor of approximately 40. Matrix and wire effects seem to be minimal. Sample throughput may be substantially improved with this system. A Tungsten (rather than Tantalum) wire loop is necessary.

1.2.2. MAIN BODY.

We speculated that the wire loop-DSID technique would provide the same advantages for ICP-MS that it does for ICP-AES for liquid samples. Probably solid samples can still be run more appropriately with an ICP-AES system due to the possibility of clogging the mass spectrometer. It is quite conceivable that the two spectroscopic methods can be combined for certain applications.

In conjunction with Mr. David Boomer of the Inorganic Trace Contaminants Section we designed, built and installed a DSID device for use on their new SCIEX Elan 250 ICP-MS. It was felt by Mr. Boomer and myself that this was an important and logical path for the research to take while we were actively working with the wire loop DSID system. The initial results are very promising. Even with the first generation of software, which is not designed for transient signals, we were able to achieve an order of magnitude improvement in the detection limits. Precision was approximately 6% RSD. As we stated in the previous section, this can certainly be improved upon with an automatic sampling-injection system.

No matrix or mass spectral overlap problems were encountered with the W wire. Ta is unsuitable for horizontal operation since the temperature is just sufficient to allow the wire to soften and bend. Since the liquid sample is dried prior to insertion, the analyte enters the plasma without the water vapor which normally accompanies it with conventional sample introduction by nebulization. This leads to an unexpected benefit. By eliminating the water from the plasma during the sample vaporization, the formation of the oxides of the elements is severely reduced. These oxides are the source of significant mass spectral overlaps in conventional ICP-MS (e.g. ArO on Fe). This is especially important with the molecular O₂ overlap with S³². Sulfur is an important element which can be difficult to determine at low levels by optical methods. The elimination of water also means that more energy appears to be available for ionization and sample breakdown. The temporal resolution may prove advantageous in cases where isotopes overlap.

1.3. WIRE LOOP STEPPER MOTOR DRIVEN DIRECT INSERTION DEVICE.

1.3.1. ABSTRACT.

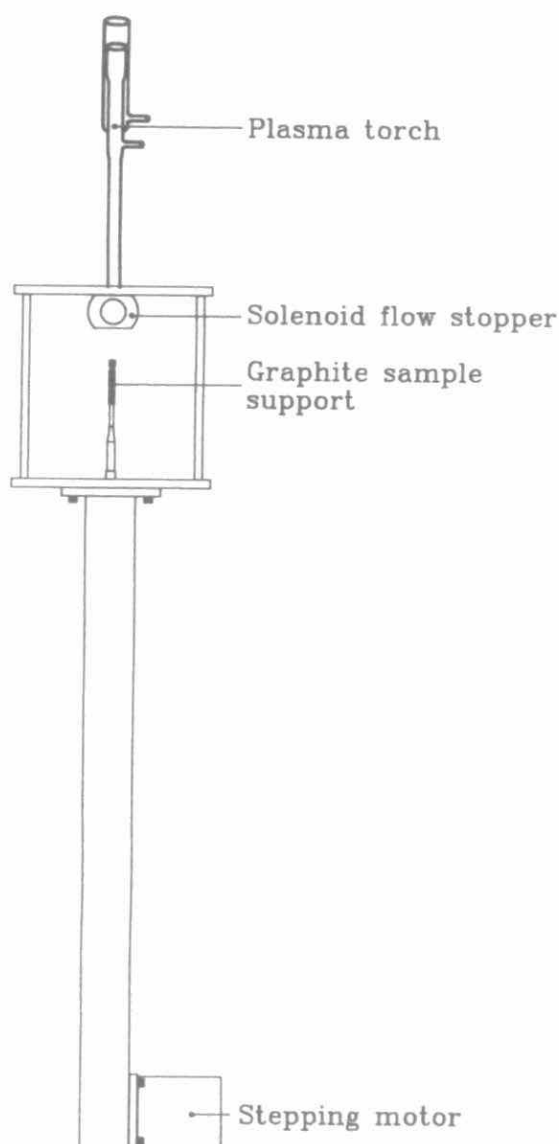
A stepper motor driven DSID was developed. The system provides RS-232 serial control and can be used with or without supporting computer. Sufficient speed is provided for wire loop insertion. The flexibility allows the system to be used for solid sample ashing, drying and insertion.

1.3.2 MAIN BODY

The pneumatic DSID performed well for desolvated liquid samples applied to the wire loop sample support. It did, however, suffer from two major deficiencies for solid sample analysis: the ejection of the sample by the sudden insertion into the plasma and the lack of fine control of the movement. Drying and ashing steps might be necessary for solid samples and, since the heating is achieved by moving the sample support closer to the plasma, fine control of the velocity and position is required. A stepper motor design of the DSID was developed (Figure 1) to provide the additional control necessary for the solid samples while still providing the speed required for the wire loop DSID work. The direct drive configuration of the stepper motor DSID design using a 1 inch sprocket provides linear velocity up to 15 inches/second. This velocity was demonstrated by experiment to be sufficient for the wire loop DSID work.

The fine control of the position of the sample support enabled the cooling of the sample support in the lower section of the plasma torch, thus eliminating the need for a sealed sample chamber as was required with the pneumatic DSID. A computer controlled solenoid driven port now ensures the sealing of the base of the torch while the sample support is retracted. The 'wide open' sample area makes the sample support very accessible for manual or automated sample application. A simple automatic liquid sample deposition device was developed for use with the wire loop DSID and provides significantly improved reproducibility and sample throughput. A versatile robotic (or micro-robotic) manipulator for the mounting of sample supports containing solid samples is presently being designed.

FIGURE 1



2. SOLID SAMPLE ANALYSIS

2.1 ANALYSIS OF HAIR.

2.1.1. ABSTRACT:

The DSID was demonstrated to be capable of the direct analysis of single strands of hair without ashing or other sample preparation. The precision is element dependent. The accuracy is within 40% and may be better.

2.2.2. MAIN BODY.

A number of hair samples were analyzed by a variety of accepted procedures. The results were in good agreement. We now have three standardized hair samples and well developed procedures for new samples.

Our initial work with conventional electrodes was unsatisfactory. A new electrode with a deep well was developed. This allowed the insertion of one or several strands directly into the plasma without any ashing or drying step. Our calibration was carried out using water samples. This is essentially ideal due to the simplicity of water standard preparation (or commercial availability). The peak areas and time behavior of a given element in a hair sample appear to be identical with that in an aqueous sample. This drastically simplifies analysis procedures and requires only a simple standard preparation step.

The precision of the technique was element dependent. Copper had a relative standard deviation (RSD) of 30 % while Zn had an RSD of 10 %. The literature demonstrates that this is to be expected as Cu concentration is often dependent on the distance from the scalp, while Zn is relatively independent. The Cu precision can probably be improved by the insertion of multiple strands in a given insertion. The Zn RSD is typical of the DSID device and can only be improved by multiple insertions.

The accuracy varies from sample to sample. It appears that we had trouble with certain elements with one donor, but other elements with another donor. With the low number of donors it was impossible to make more emphatic statements. In no case was the error worse than 40%. If an average correction value is used, since all values were high, the maximum error drops to 20%. This will be acceptable for some work, e.g. toxicology, but may not be adequate for others. Certainly this is a marvelous rapid survey technique as it stands. More work needs to be done to clarify the accuracy problem.

2.3. SEDIMENTS.

2.3.1. ABSTRACT:

Solid sample studies of estuarine sediments have been initiated recently. Detection limits appear to be adequate for the qualitative identification of toxic elements, but the accuracy and precision is not adequate yet for quantitative work. Various instrumental problems are discussed.

2.3.2. MAIN BODY:

The sediment types recommend by Mr. Boomer of the MOE were acquired. These include BCSS-1, MESS-1 and SRM 1646 . We initiated our work with these after the stepper motor DSID (section 1.3) was completed. After initially profiling the samples for detection limits for the toxic elements (see Table II), we began work on stabilizing the signal level for Cu. Copper is an element which we commonly study and is in relatively high concentration in all these samples. Once we have stabilized with Cu, then we expect that we will have improved our precision and accuracy for all the elements. Our precision at this stage (two months of work) is still at such a level that we can not make substantial statements about the need for special standards. Our hope is that graphite based standards, containing oxides of the analyte, will work, however the time behavior is drastically different between each of the sediments and the graphite standards. We also believe at this time that a certain amount of our precision problems arises due to inhomogeneity problems. Since we only use 1 to 10 mg per insertion, this is quite possible. Our own "home made" standards which are more finely ground (about the same mesh as the sediments) have provided higher precision.

Table II. Detection limits in solid sediments estimated for MESS-1.

Element	Certified concentration $\mu\text{g/g}$	Estimated detection limit based on 2 mg sample $\mu\text{g/g}$
Cu	25.1	0.8
Cd	0.59	0.2
Zn	191	8
Pb	34.0	2
Mn	513	32

We believe that it may also be necessary to use either mixed gases or fluxing agents when preparing the samples. In the matter of sample preparation we believe that the processes can be conveniently and very effectively automated using a commercially available robot. This could be quite cost effective since the same robot can be used for dc arc and other sample preparation procedures as well as incorporated into the DSID process.

Rapid background correction is required for samples which generate a large amount of stray light, and this is discussed in the next section.

3. DETECTION SYSTEM REQUIREMENTS FOR DSID OPERATION

(REFRACTOR PLATE BACKGROUND CORRECTION)

There are two methods of background correction presently available on commercial direct reading spectrometers. These are vibrating slits and refractor plates. Vibrating (moving) slits are limited to relatively slow movement and actually view a slightly different portion of the plasma when spectrum shifting. This is not a problem with conventional sample introduction system, because the sample is uniformly distributed though a large central aerosol channel in the plasma, however this is not satisfactory with the DSID system.

Refractor plates shift the entire spectrum after it has been imaged onto the entrance slit and consequently are adequate for this type of work. In the Thermo Jarrell-Ash line of spectrometers these are mounted on galvanic drives and consequently are capable of rapid movement, though the normal software does not provide this capability. On the system that we are acquiring, we will be working with Thermo Jarrell-Ash to develop software and hardware suitable for the high speed acquisition of spectrally background corrected signals.

ELECTROTHERMAL VAPOURIZATION FOR SAMPLE INTRODUCTION INTO A PLASMA
FOR PLASMA SOURCE MASS SPECTROMETRY

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1.0 INTRODUCTION

Inductively coupled plasma source mass spectrometry (ICP/MS) is still in its infancy as a method of chemical analysis. A 1985 comprehensive review of ICP/MS by Douglas and Houk [1] lists 54 references. Since this report has been written there have been between 20 and 30 other reports published in this field. The rapidly rising rate of appearance of publications mirrors the commercial introduction of ICP/MS equipment three years ago.

Most workers in this field use conventional nebulization (concentric and cross flow) systems for sample introduction into the ICP. Using this approach detection limits up to 2.5 orders of magnitude better than flame atomic absorption spectrometry (AAS) and plasma optical emission techniques have been reported. These powers of detection approach or, in some cases, equal furnace AAS. Despite these excellent powers of detection, for many investigations (e.g., environmental, biological and clinical), it would still be a distinct advantage to achieve lower values.

The direct injection of μl aliquots of sample into the plasma in plasma optical emission spectrometry has yielded at least 1 order of magnitude better detection limits compared to conventional nebulization. Such an approach has the advantage over conventional nebulization that small samples (volume limited), highly viscous samples and possibly solid samples can be run. There are a variety of ways this can be done [2]. These include (1) direct insertion of sample along the sample uptake channel using wires or graphite rod, (2) micro-arcs or sparks within or outside of the torch system, and (3) electrothermal vapourizers (ETV). We have chosen to investigate the utility of number 3.

"Potential" advantages over the other two direct insertion methods include:

- (1) Use of a larger sample size.
- (2) Opportunity for matrix modification.
- (3) Closely controlled, multiple heating cycles.

Early results with an Re filament and a graphite filament ETV are reported below.

Another, related, investigation was also undertaken. Mass spectrometry has been a very useful and important detector for gas chromatography since 1957 [3]. In this application the MS and GC are interfaced so that the atmospheric pressure GC effluent is transferred through a region with a large pressure drop until the desired constituent enters the MS at a pressure of less than 10^{-6} torr. The introduced effluent is also subjected to an appropriate method of ionization prior to mass separation and detection. The inductively coupled plasma could be used as an excellent ion source for this application. In this way the mass spectrometer becomes an element specific detector for chromatography. There is an urgent need for environmental, toxic metal-organic compound data and the ICP source mass spectrometer should be an excellent detector for chromatography in this application. This detector is isotope specific and thus would, in comparison with other important, but only, element specific detectors (AAS, AES, AFS), be capable of producing important additional information on the occurrence of toxic metal organic species in the environment. In the following, the first recorded (preliminary) data is given, outlining the use of ICP source mass spectrometry as an element specific detector for gas chromatography.

2.0 EXPERIMENTAL

2.1 Equipment

The ICP/MS apparatus used was designed by Douglas and French [4] at the Institute for Aerospace Studies, University of Toronto. Further development by Sciex, Thornhill, Ontario, Canada, has resulted in a commercial product, the ELAN.

The electrothermal vapourizer (ETV) was developed for the purposes of this investigation and its design and optimum operation are described in detail previously [5]. A summary of its characteristics are in Table 1. The filament consists of 0.05 mm thick Re metal ribbon (available from REMBAR Laboratory, New York) of sample capacity 5 μl , which can be resistively heated using a crude home-made power supply. This is constructed from a 1 KW transformer and lab variac. Maximum output capability is 2.5V and 30A. The filament is encased in a glass dome which is sealed to a metallic base by an "O" ring. Argon is flowed through the device to carry the vapourized sample, via the Tygon tubing, into the ICP.

Table 1. Summary of ETV Characteristics

Volume of glass dome above filament	5 ml
Inert gas introduction	Tangential at base (~4 l/min) laminar flow (Reynolds number of system is 150)
Filament	Re metal (central sample dimple 1 mm deep by 5 mm diameter - 5 μl capacity)
Maximum temperature	1900°C (above this severe plasma loading occurs due to Re loss)
Particle size of aerosol	0.5 to 1.0 μm (Zn)
Transport efficiency* at 4l/min (%)	Cd(85), Pb(83), Cu(84), Fe(85), Ni(83)

*Greatly improved compared to previously listed devices [2].

The ICP was obtained from Henry Radio (Model 2500). Output powers of up to 2 KW are achievable. This device must be operated and adjusted for the most part manually. A standard cross-flow nebulizer for solution sample introduction, when required, was used for initial optimization of ion optic and MS parameters for each element.

Mass selection is achieved with a quadrupole mass spectrometer. The quadrupole is particularly well suited for mass analysis using a plasma source. The 10^{-4} to 10^{-5} torr operating pressures tolerated by quadrupoles reduce the pumping speed requirement and allow the use of relatively large orifices. Fringing field effects which are encountered at the entrance and exit of quadrupoles are minimized by the use of short sections of AC only quadrupole rod sets. This provides improved resolution capability.

The interface of the plasma to the ion optical system consists of a sample orifice approximately 0.4 mm in diameter. The orifice is a nickel conical structure mounted on a water cooled copper flange. Immediately behind the sample and separated by a region ($12 \times$ the diameter of the orifice) which is pumped mechanically to about 1 torr, is a skimmer orifice 1 mm in diameter.

A cryogenic pumping system, supplied by CTI-Cryogenics Ltd., is capable of pumping the mass spectrometer down to a pressure of about 2×10^{-5} torr during operation.

A Model 9176 x-y recorder, available from Varian, was employed for display of spectra. Operating conditions for the equipment are given in Table 2.

Table 2. Instruments and Operating Conditions

Operating Conditions:	
Forward power	1.25 KW
Reflected power	0 W (as close to this as possible)
Coolant flow	14 l/min
Auxiliary flow	0.7 l/min
Carrier flow	2 l/min
Drying	100°C for 20 sec
Ashing	(400-800°C) - depends on type of sample and element
Vapourization	(up to 1800°C)
Clean-out	(2500°C)

A Fisher Scientific Series 2400 temperature programmable oven was used. The experimental parameters are given in Table 3.

Table 3. Gas Chromatographic Experimental Parameters

Column	3% OV-1 on Chromasorb W (80/100 mesh)
Gas flow rate	Argon at 8 ml/min oxygen (into transfer line) at 3 ml/min
Injector temperature	250°C
Temperature program	Isothermal 60°C to end of peak (1). Isothermal 250°C to end of chromatogram.
Transfer line (stainless - 0.5m length) temperature	Just high enough to ensure no breakup of peak ~200°C (using Variac and heating tape).
Interface	Transfer line connector (Swagelok) to base of ICP torch

2.2 Reagents

All metal salts, solvents and acids used were Reagent Grade quality or better. Atomic absorption standard solutions of metals 1 mg/ml in concentration, were obtained from Fisher Scientific.

Calibrating solutions ($\mu\text{g/ml}$) were prepared by dilution of the 1000 $\mu\text{g/ml}$ standards. For preparation of $\mu\text{g/ml}$ calibrating solutions the 10 or 1 $\mu\text{g/ml}$ calibration solutions were diluted. All calibration solutions were single element and were made to contain 1% nitric acid upon final dilution. The concentration range covered was 1 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$.

National Bureau of Standards (Washington, D.C.) Standard Reference Samples were used to test the methodology. Samples analysed were Orchard Leaves (SRM 1571), Oyster Tissue (SRM 1566) and Pb Isotope Standards SRM 981 and SRM 983.

The following Sn compounds were prepared by pentylation using the method described by Maguire and Huneault [6] - Me_3SnPe , Me_2SnPe_2 and MeSnPe_3 (PAe is methyl and Pe is penty).

2.3 Proposed Procedure for ETV Sample Introduction

(The system must be pumped down to 1 torr in the interface region and 2×10^{-6} torr in the mass spectrometer. This takes approximately 2.5 hours from a cold start.) Turn on all electronic systems and ignite the plasma. Aspirate a 1 $\mu\text{g/ml}$ solution of the element to be determined. Optimize the ion optic components so that maximum analyte signal is obtained. The instrument must then be left operating until any analyte signal drift has stopped, a minimum of 1 hour. At this point the system is changed from nebulizer to ETV operation.

Orchard Leaves (SRM 1571) and Oyster Tissue (SRM 1566) samples are dissolved in closed Teflon digestion vessels by the method of Brzezinska et al [7]. A mixture of HNO_3 , HClO_4 and HF is used. The acid content of the solutions after dilution to final volume is 1% in HNO_3 .

Sample and standard solutions are analyzed as follows. Remove the glass dome from the ETV. Pipe a 2 μl aliquot of sample or standard solution

onto the Re filament. (The auxiliary Ar flow, 2 l/min , of argon to the plasma bypasses the ETV during this operation.) Replace the glass dome and clamp it tightly onto the ETV base using the toggle clamps. Divert the ETV Ar flow of 2 l/min by means of the 3-way stop cock through to a fume hood vent. Dry the solution at about 100°C until the drop of liquid has been completely evaporated (visual observation). Char the standard or sample, if necessary at 400° to 800°C for 30-60s.

Using a 3-way stop cock switch the ETV Ar flow (at 2 l/min) to pass into the plasma. Then turn off the auxiliary Ar flow. Vapourize the sample at up to 1800°C . Record the transient signal obtained. Signal integration must be used in computing elemental concentration in Orchard Leaves and Oyster Tissue solutions.

Calibration is done by the method of standard addition as follows. Run the sample alone, as outlined above. Then add 2 μl of suitable concentration of calibrating solution to the Re filament and dry as indicated above. Remove the glass envelope and add 2 μl of the sample solution and dry again as detailed above. Then vapourize the material on the Re filament according to the above procedure. This procedure is then repeated using a 2 μl spike of calibrating solution which is twice the concentration of the first spike. Again it is important to emphasize that signal integration must be employed in computing the signal values for these runs.

2.4 Proposed Procedure for Gas Chromatography

Turn on argon and oxygen gas flows to the flow rates given in Table 3. Preheat the injection valve and transfer line. Inject 10 μl of the sample solution into the GC using a gas tight syringe. Initiate the thermal program given in Table 3. Record the signal over a period sufficient for

the complete removal of compound electron is Me_3SnPe , Me_2SnPe_2 and MeSnPe_3 . Measure the peak area of each compound.

2.5 Interferences (ETV Investigation)

Mass spectral overlap interferences are much less severe than optical spectral overlap, particularly when plasma sources are employed. In ICP/MS the most serious interference problem appears to be ionization suppression.

2.5.1 Ionization Suppression and Vapourization Interferences

All elements, present in significant amounts in a sample, can potentially cause ionization suppression problems. Those with ionization potentials below 8 eV are of greatest concern in this regard.

When the matrix substances are present to the point that they significantly increase the electron density in the ICP or significantly reduce the argon reagent ion levels, they can affect the analyte ionization and hence the signal. Since the typical electron density or total ambient ion density in the axial channel of the plasma is 10^{15} cm^{-3} [5], when the number density of easily-ionizable matrix atoms in the axial channel approaches this value, the degree of ionization of the analyte atoms becomes much lower than that without the matrix elements. The amount of an easily ionized element which will cause this problem has been found to be 1 μg (Na). This matrix effect in the ICP is often termed a plasma loading interference.

It is important to separate out the plasma loading interference from other matrix effects of the ETV/ICP/MS combination. To do this an ETV was designed similar, in all respects, to the single filament one [5], except that 2 filaments were installed side by side. In this way separate aliquots of matrix and analyte could be vapourized simultaneously. Thus 1 ng analyte

elements and 1 μg matrix elements could be vapourized simultaneously from two adjacent filaments and their signals compared with those from vapourization from the same filament.

Results of such investigations for a variety of analyte and matrix elements have been previously obtained and will appear in detail in an upcoming publication [8]. For the elements of interest in this study, Cu, Pb and V, both vapourization and plasma loading interferences were identified when ng levels of analyte were vapourized in the presence of μg levels of matrix elements.

2.5.2 Compound Formation

Compound formation on the filament volatilizer is suggested by the appearance of multi-peaked signal tracings. (Another possible cause of double peaks could be that the analyte element is absorbed on the filament surface in 2 different states having different desorption energies.) Multi-peaked signals have been noted by many researchers, particularly in furnace atomic absorption work. In some cases the compound formed can be identified from the analyte appearance temperature. In practice, low heating rates are helpful in studying this phenomenon because they permit more clearly-resolved peaks. Conversely, very high heating rates tend to obviate this effect, a finding which is very important for quantitative analysis using the ETV/ICP/MS system.

Examples of compound formation involving the analyte and the Re filament (e.g., Se) and the analyte and sample matrix constituents (e.g., V with Cl) have been noted. In addition, Cl (a common matrix constituent) interaction with the Re filament was a serious problem. (See acid-anion interferences below.)

2.5.3 Anion Interference

Real sample solutions at their final volumes must contain 1% acid concentration to ensure stability of trace elements in solution. Thus anion(s) associated with acid(s) are present at relatively high concentration. Acid effects have been demonstrated for conventional ICP/MS systems (i.e., those which involve aspiration of the sample solution into the ICP). Effects, particularly from P, S and Cl, containing anions can be severe [9]. Sample introduction by ETV has a potential advantage over such systems. With the ETV the sample can be dried and ashed prior to introduction, thus allowing removal of excess acid anion. Also if matrix modification is used the identity of the remaining anion (i.e., that bound to the analyte and remaining matrix) can be changed if desired. Because of the well known adverse properties of phosphate anion on the ICP/MS system [9] no investigation of this anion was attempted.

The effect of different anions on copper signal was studied, first, when the molar ratio of Cu to anion was 1:1 no adverse effects on the copper signal were noted for chloride, nitrate, sulfate or acetate. When Cu solutions were made up with 3% and 6% acid (concentrations commonly used in ICP optical emission) serious anion effects were found for both concentration levels (greater detail will appear in Park et al [5]). Results show that both peak height and peak area reductions occur. A greater problem occurs with the higher acid content (6%). In all cases sulfuric acid gave the largest signal reductions. Depending on the element varying magnitude of signal decrease was noted for the other acids.

In many cases the magnitude of signal reduction also varied with the final Re filament temperature. For example, with nitric acid a lesser Cu peak height reduction occurred for higher filament temperatures. The opposite was true with hydrochloric acid.

The observed peak height and peak area reductions may be due to two factors:

- (1) The acid-anions lower the desorption rate (reduces peak height and broadens peak).
- (2) The acid-anions cause an increase in Re loss from the filament which results in plasma loading (decreases peak area).

One of the potential advantages of the present system over direct insertion using a wire loop is the capability for controlled temperature ashing investigations. Preliminary ashing investigations made to date, without a controlled temperature ramping step show an appreciable improvement (up to 100%) in signals obtained when sulfuric acid was the major acid anion. Lesser improvements were noted in the case of other acid anions. Future studies involving matrix modification and slow temperature rampings to the final ashing temperature will be done. This approach should be particularly useful in removing chloride (e.g., add NH_4NO_3 to remove chloride as NH_4Cl).

The following generalizations are important at the present state of ETV methods development:

- (1) The anion effects on the analyte signal are negligible when the anion concentration is not significantly higher than the analyte concentration. Therefore, for maximum sensitivity, acid solutions of the lowest possible concentration should be used in preparing the sample solutions.
- (2) To avoid gross systematic errors when calibrating by the standard addition technique, both sample and reference solutions should be made from the same kind of acid solutions and have the same final acid concentration.

It is of interest to pursue the possible use of high levels of chloride to improve the volatility of metals during the volatilization step (many metals form chlorides which have boiling points in the 0 to 800°C range). To investigate the possibility of signal enhanced in the presence of chloride, the levels of this element must be much higher than would be achieved at normal acid matrix concentrations. In this regard μl aliquots of solutions of MgCl_2 at varying concentrations up to saturated solution levels were added to the aliquot of metal on the Re filament. Results show that a significant improvement in peak height for all the elements studied (Pb, Cu and V) may be achievable. However, the ultimate magnitude of this improvement was masked by a deleterious plasma loading effect resulting from the increased loss of Re from the filament. Very preliminary data obtained from a graphite fibre filament suggests that this approach may be quite fruitful. Hence future investigations with magnesium chloride solution on metal volatility from the graphite fibre filament will be made.

A new programmable ETV power supply is almost completed. This device will allow the use of a wide range of temperatures and heating rates for drying, ashing and vapourizing. In this regard our experiences with the present manual adjustments of temperature and only one choice of heating rate suggests that problems of sputtering and popping of samples and incomplete matrix modifications obtained during drying and ashing should be largely overcome with the use of the programmable supply.

2.5.4 Matrix Spectral Interferences

When solution nebulization is employed several potential interferences involving species of oxygen, nitrogen and hydrogen are possibly in the mass spectrum. The use of an electrothermal vapourizer minimizes such a problem.

The most severe mass spectral interference noted, to date, in these investigations was Zn^{64} on Cu^{63} and Cu^{65} . With the present instrument it was not possible to use a resolution good enough to eliminate the problem. Thus, under best conditions, it was found that 1 $\mu\text{g}/\text{ml}$ Zn generated a signal equivalent to about 62 ng/l of Cu.

2.6 Calibration (ETV Investigation)

If possible, it was desirable to construct a calibration graph from signals obtained by running simple dilute acid calibrating solutions. The signals obtained from the samples are then compared with those obtained from the calibration graph to give the solution concentration for the sample. It was found, however, that such an approach yielded erroneous results for several types of samples. Thus the method of 'standard additions' was used for quantitative work. Signal integration was used for all determinations.

2.7 ETV Operational Parameters

The ETV presently being used has no capability for adjusting the temperature ramp during drying, ashing and vapourization cycles. Thus as touched on above, considerable problems with sample decrepitation were encountered.

The drying step in particular is very sensitive to the speed of temperature rise. It is desirable to employ a slow (20 to 30 sec) ramp from room temperature to 100°C to ensure no sputtering. In the work to date, sputtering was frequently noted during the dry cycle.

The ashing step provides the opportunity to remove troublesome matrix ingredients. Little success has been achieved to date, in this regard, again mainly because the present power supply was so inflexible. Experiments with matrix modifiers such as NH_4NO_3 to remove chloride were

largely fruitless. Losses of volatile analyte and analyte halide were frequently encountered even at power supply adjustments which should give filament temperature below critical volatilization temperature. This was probably due to momentary temperature overshoot.

The ash step is important for the removal of organic matter. Under the present conditions encrustations on the Re filament of black residues were common even after the ash step. To aid in the ashing of organic material, investigations were carried out to determine if oxygen could be utilized in the carrier gas stream, during the ash step. Unfortunately, this resulted in a rapid deterioration of the Re filament. The use of oxygen during ashing will be investigated further with the graphite filament.

Flow rate of the carrier gas through the ETV is a very critical parameter. For the elements studied to date, a value of 4.0 l/min was found to be optimum. For example, when the flow rate reached 5.5 l/min , the signal has been reduced to less than 10% of its optimum value. Surprisingly, with the 3 dome sizes (increasing volumes above the filament by 2 and 6 times), there was no appreciable change in the optimum (4.0 l/min) and limiting (5.5 l/min) flow rate requirements.

The permissible compositions of the filament are of great importance. Metal filaments have the advantage that the power supply can be relatively simple and small in output. In addition, it is easy to cut and form them into a useful geometry. Of the suitable metals only Re proved to combine good forming characteristic with durability under most conditions. However, when volatilization temperature of 1900°C and above are required even Re is not satisfactory. For this purpose graphite has been chosen and details of its characteristics and utility will appear in the next report.

2.8 Plasma Power

An incident power of 1.25 KW was used throughout this work. At higher powers the analyte signal increased significantly but this was counter-balanced by an increase in background signal.

2.9 Operation of ICP/MS for GC Detection (Chromatography Investigation)

The ICP was run in a very similar manner to that used for the ETV work. However, when argon alone was used in the chromatograph and as the carrier gas, a large carbon buildup occurred on the outlet of the sample uptake channel. This resulted in very poor detection limits and deteriorating plasma stability. To overcome this problem, it was necessary to add oxygen to the carrier gas. This was done by installing an oxygen inlet in the heated transfer line. The oxygen flow rate was adjusted to just completely eliminate the carbon deposit (10-20% of total gas flow). The oxygen flow rate required depended on the identity of the solvent being used.

A plasma incident power of 1.25 KW was found to be optimum for this application. The tuning conditions for the oxygen-argon plasma were quite different from those for a purely argon plasma.

3.0 RESULTS

3.1 Determination of Cu and Pb in SRM's (ETV Investigation)

3.1.1 Analysis of Solutions

A variety of biological and environmental standard reference samples were analyzed using the proposed procedure. Copper and lead results are given in Table 4. Vanadium determinations were also attempted. However, further investigations of the graphite filament vapourization will be necessary before quantitative results can be obtained for vanadium.

Table 4. Values Obtained for Cu and Pb in SRM's

MESS-1	21±2 (25±3.8)*	34±1 (34±6)
BCSS-1	22±1 (185±2.7)	21±2 (23±3)
Tomato L	10±2 (11±1)	6.1±2 (63±0.3)
Tort 1	431±39 (439±22)	11.0±2 (10.4±2)
River Sed.	112±6 (109±19)	714±35 (714±28)
Coal Fly Ash	96±10 (118±3)	70±3 (72.4±0.4)
Urban Particulate	624±120 (609±27)	TH

*Certified value. TH - too high for ETV.

These results are preliminary as significant improvements are to be expected with use of the programmable power supply. In spite of this, the results obtained for Cu and Pb show an acceptable agreement with the certified values in the majority of cases.

Several other elements have been determined in a few of these samples. However, these results are not recorded here because the list of samples

analyzed is very incomplete and there are few and, in many cases, no certified values for these elements in the standards.

3.1.2 Direct Analysis of Solids

The ultimate goal of this research is to develop the capability to directly analyse solids (i.e., without need for chemical sample preparation). In this regard, it was of interest to attempt an early run to look for major problem areas which could be developing.

NBS Bovine Liver Powder was slurried with water and a 2 μ l aliquot dispensed onto the Re filament. The following thermal program was employed: dry at 150°C (30 sec), ash at 800°C (30 sec) and vapourize at 1950°C (3 sec). During the dry and ash stages, decrepitation of the sample occurred. Also the peaks obtained for Cu and Pb were broader than is desirable. This means that ashing must be more complete and that the vapourization temperatures should be higher. Using the Re filament it is not possible to use a higher temperature. Thus further work on direct solids analysis must await the programmable power supply and further investigation of the graphite filament.

3.2 Separation and Determination of Tin Compounds (Chromatography Investigation)

At this point no results have been obtained on real samples. Synthetic mixtures of the three tin compounds of interest (Me_3SnPe , M_2SnPe_2 and MeSnPe_3) were chromatographed using the proposed chromatographic conditions and procedure. Chromatographic peaks showing good resolution of the 3 compounds were obtained. Table 5 lists the retention times and best detection limits obtained at this time.

Table 5. Retention Time and Detection Limit Data

Retention Time (sec)		Detection Limits (ng)
Me ₃ SnPe	74	6
Me ₂ SnPe ₂	95	16
MeSnPe ₃	134	10

Detection limits obtained by Chau et al [10] were 0.1 ng for each compound. Because of problems with our prototype ELAN, the signals obtained in any investigation are between 10 and 100 times poorer than the commercial instrument. Taking this into account our detection limits for these preliminary investigations are quite good for this early stage in the investigation.

4.0 CONCLUSIONS

- (1) Two sample introduction systems for ICP/MS were studied:
 - (a) An electrothermal vapourizer (ETV) was evaluated for the introduction of liquids and solids. The elements Cu, Pb and V were investigated.
 - (b) A gas chromatograph was interfaced to the ICP/MS and the separation and determination of alkyltin compounds was studied.
- (2) ETV results:
 - (a) An Re filament was used in the determination of Cu and Pb in a variety of standard reference samples. (Vanadium could not be determined using the Re filament because of its low volatility and the severe plasma loading problem which occurs when Re is used at temperatures above 1900°C)
 - (b) The results obtained for Cu and Pb in the standard reference samples were, for the most part, in acceptable agreement with certified values.
 - (c) A carbon filament is being investigated for the determination of Vanadium. Early results suggest a marked improvement in signal shape and magnitude for this filament.
 - (d) Ionization suppression interferences and anion interferences were found to occur in the ETV approach. However, controlled programs for drying and ashing (used together with matrix modifiers) which will soon be available to us will allow suppression to some degree of these problems.
 - (e) Mass spectral overlap interferences can also be encountered with this approach. However, since air and water are excluded from the ETV sample introduction system, a simpler pattern of such interferences is obtained compared to nebulization sample

introduction. The most serious spectral overlap problem encountered to date was the slight overlap of Zn^{64} with Cu^{63} and Cu^{65} . A correction was made for this problem.

(f) Operational parameters and ETV Figures of Merit:

- Ar flow through ETV chamber should be 4.0 l/min.
- Incident plasma power 1.25 KW (reflected power near zero).
- Sample size, up to 5 μl (of slurried solid or sample solution).
- Re metal filament can be used to 1900°C, carbon filament to 2500°C (estimate).
- Method of standard additions used for calibration.
- Transport efficiency for Cu and Pb better than 80% (solutions of analyte plus dilute acid).

(3) Gas chromatographic results.

(a) Compounds separated were Me_3SnPe , MeSnPe_2 and MeSnPe_3 (Me = methyl and Pe = pentyl) using a 3% OV-1 on Chromasorb W (80-100 mesh) column.

(b) Operational parameters of importance are:

- Flow rates: Ar - 8 l/min
 O_2 - 2 l/min (introduced in transfer line)
- Temperatures: Injector, 250°C
Column, 60°C to end of peak (1) and 250°C to end of chromatogram
- Plasma incident power, 1.25 KW, reflected power near zero.

(c) When Ar is used alone in the system, carbon deposits, which trap Sn, occur in the torch and orifice. Thus oxygen must be introduced into the transfer line.

(d) Results obtained:

- Elution order of compounds MeSnPe_3 , Me_2SnPe_2 and Me_3SnPe .

- Retention times in sec and (detection limits in ng) 74(6),
95(16), 134(10).

5.0 ACKNOWLEDGEMENT

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DEVELOPMENT OF SELENIUM ANALYSIS METHODOLOGY AT NG/L LEVELS
FOR SOFT WATERS SUBJECT TO ACIDIC PRECIPITATION

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ABSTRACT

Our studies of nutritional factors important in controlling the composition of phytoplankton populations have indicated that some phytoplankton species in softwater lakes subject to acidic precipitation require the element selenium for growth. Some of these phytoplankters produce undesirable blooms. The levels of selenium required by these species is low. However, the meagre data available on Se concentrations in softwater lakes indicate that Se levels may be low enough to limit the growth of some phytoplankton, and that Se inputs from acidic precipitation, a known source of Se, may affect the composition of these communities. Because of the dearth of data on Se levels, it was decided to apply a sensitive method to analysis of this element in soft waters. In addition the known effects of Se in ameliorating some of the toxic effects of Hg suggest that Se may be important in soft waters for other reasons.

Atomic absorption methods as conventionally applied yield a detection limit in the $\mu\text{g/L}$ range. This is inadequate for the studying the levels normally encountered in softwater lakes. A gas chromatographic method was applied and allowed detection at the low ng/L level. Several new features of this method have not been used previously in analysis of Se in natural waters. These include application of capillary chromatography to analysis of the selenenol derivative (of 4-nitro-o-phenylenediamine) in soft waters, and a single flask derivatization procedure which reduces handling and potential contamination. Retention time of the selenenol peak was about 1.8 min with total analysis time of 5 min, at an operating temperature of 250 °C. Preliminary data indicate that Se concentrations vary with season and depth and are typically less than 1 $\mu\text{g/L}$ in softwater lakes. The method can also be used to study the distribution among the various species (Se^{2-} , Se^{VI} and organoselenium).

INTRODUCTION

The element selenium was discovered by J. J. Berzelius, a Swedish chemist, who in 1818 scraped it from the walls of vats in a sulfuric acid plant (Oldfield 1981). This early observation anticipated our current interest in Se in relation to the emissions of SO_2 which give rise to acidic precipitation. It has long been known that Se accompanies sulfur as an air pollutant emitted as a result of the combustion of coal (Cooper et al. 1974). This is the single most important source of atmospheric Se, contributing more than 60% of the Se emitted to the atmosphere, exceeding the amount from metal smelting (primarily copper) the second most important source (National Academy of Sciences Committee 1976). The comparison between total natural emissions and anthropogenic emissions of Se is poorly explored, but the best current estimates indicate that the emissions due to industrial activity (e.g. coal burning and copper smelting) exceed natural sources by at least 5-fold or as much as 34-fold (Galloway et al. 1982). Also, Se (along with As) is particularly enriched

in power plant emissions compared to other elements (Chadwick and Lindman 1982). Evidence from sediment cores indicates that an increase in Se deposition, presumably due to atmospheric emissions has occurred since the 1950's (Heit et al. 1981). Most inorganic Se is probably insolubilized in the sediments (Turner and Rudd 1983). However, some may be resolubilized in an organic form (Cutter 1982).

Our specific interest in Se was stimulated by our observation that Se is absolutely required for growth in culture by Chrysochromulina breviturrita (Wehr and Brown, 1985), a phytoplankter which causes undesirable blooms in lakes subject to acidic precipitation. Even at 100 ng Se/L yield was doubled over the controls. Also experiments at the Experimental Lakes Area (Kenora, Ont.) indicated that addition of electrolyte grade sulfuric acid (contaminated with Se) to lakes resulted in a C. breviturrita bloom.

A review of the literature on Se requirements by phytoplankton revealed evidence for its essentiality in several marine species of Chrysochromulina (Pintner and Provasoli 1968), a freshwater dinoflagellate Peridinium cinctum fa. westii (Lindstrom and Rodhe 1978, Lindstrom 1983) and a freshwater diatom Stephanodiscus hantzschii var. pusillus (Lindstrom 1983). Subsequent subculture in Se-free media resulted in no growth (Wehr and Brown 1985). The results significant in the laboratory context. However, the key issue is the relation between Se and phytoplankton blooms. Se from coal-fired combustion arrives at these remote areas by "episodic precipitation" (Parekh and Husain 1981), e.g. air masses which receive plumes from coal-fired power plants have sporadically high Se concentrations. We suggest that this episodic precipitation which is high in Se may give rise to C. breviturrita blooms on a sporadic basis. This would account for the unpredictable pattern of bloom occurrences observed by Nicholls et al. (1982). To test this hypothesis, environmental concentrations must be measured and the values applied in continuous culture. The present work deals with this problem, and will enable us to assess the role of Se in phytoplankton ecology.

LITERATURE REVIEW

The major problem has been an unsatisfactory detection limit (e.g. 1 ug/L Se) at the various laboratories which sometimes do Se-analyses. The most sensitive and specific method that has been applied to water samples involves measurement of the amount of the 5-nitropiazselenol derivative by electron capture-gas chromatography (Measures and Burton 1980a,b). This method is specific for Se and has been reported to provide a detection limit of 0.8 ng/L for marine waters (Measures and Burton 1980a). Seawater Se concentrations range from 20-180 ng/L (Measures and Burton 1980a), and some data indicate that Se in fresh waters are below 100 ng Se/L (Suzuki et al. 1981). In highly contaminated

lakes within the vicinity of Sudbury, concentrations range between 100-400 ng/L (Nriagu and Wong 1983), but no data exist for less contaminated lakes. These levels are well below the level of detection by the conventional AA or ICP methods currently used.

MATERIALS AND METHODS

a. Chromatography. A capillary splitter (J & W Scientific) was installed in a Varian 3700 gas chromatograph. The column of choice was a 0.2 mm (15 m) OD fused silica column (DB-5) from J & W Scientific operated isothermally at 250 °C with a flow rate of 1.5 mL/min and a split ratio of 100:1. See also Table 1.

b. Sample bottles: Linear polyethylene (LPE) or high density polyethylene (HDPE), Nalgene 500 mL size.

c. Acid washing of glassware:

1. Soak several hours to overnight in 10% HNO₃
2. Rinse at least 5x in purified (reverse osmosis) water
3. Rinse at least 5x in glass-distilled or other high quality water.

d. Acid for preservation: 1 mL of concentrated HCl per 500 mL of lakewater (more acid may be needed for high alkalinity waters). Final pH should be within the range of 1.8-2.0 with this acid addition.

e. Filtration: (as needed for evaluation of "soluble" i. e. filterable Se). We have used primarily 0.45 µm pore size Millipore cellulose acetate filters, prerinsed 5x with glass-distilled water in an acid-washed beaker. Nucleopore (polycarbonate) filters can also be used. An all glass filtration with glass-fritted and silicone bung (or all-glass unit) is recommended.

f) Derivative formation: (adapted from Measures and Burton 1980a): The reagent is 1.0 % 4-nitro-o-phenylenediamine (Aldrich Chemical Co.) (NPDA) in 10% HCl added at a rate of 2 ml per 100 mL of sample in a 100 mL volumetric flask capped with a teflon-faced, rubber-lined screw cap (Pyrex brand). This was allowed to react in subdued light for 2 h. A 1mL aliquot of toluene (glass-redistilled) is added and the mixture is shaken vigorously 25x by hand (ca. 3 min). This single container system has a considerable advantage over previous methods (e. g. Measures and Burton 1980a) which require separatory funnels and transfers of samples with the risk of both loss of samples and contamination. Layers are allowed to separate and the toluene layer removed with a toluene-washed (10x) pasteur-pipette to a 1 dram pre-washed glass vial with a teflon-lined cap (Supelco Canada Ltd. Catalogue # 2-3288). Samples prepared in this way can be stored at least 6 months at -20 °C without degradation.

RESULTS AND DISCUSSION

a. Capillary chromatography. To our knowledge all of the previously reported work (Dilli and Sutikno 1984) has been with packed columns with their inherently lower resolution compared to capillary columns. It seemed a worthwhile objective to develop a

capillary based system for these analyses especially in view of the fact that chromatograms for most of the previously reported work have not been particularly clean.

Our first attempts with a capillary type system has been with a megabore (large internal diameter) column, J&W Scientific DB-WAX (15 m). This column proved to be a poor choice as the Se-derivative (piaselenol) and the unreacted derivatizing reagent appeared to react with the column liquid phase giving a very poor chromatogram from the view of sensitivity and resolution.

The next column tried was another fused-silica megabore column, J&W DB-1 (15 m). This column has a relatively nonpolar methyl-siloxane bonded liquid phase (similar to an Se-30 silicone gum). It provided resolution of close to the level of the packed column work in the literature. We observed, however, a small neighboring peak which appeared to interfere with quantitation of the piaselenol. Our attempts to resolve this peak from the piaselenol by temperature programming were not successful. At this point it was decided to convert the chromatograph from a packed column instrument to full split capillary configuration. An inlet splitter (J&W Scientific) was installed on the inlet end and a fused silica capillary detector insert (Varian Instruments) was installed on the detector end of the Varian 3700 chromatograph. A comparison of chromatograph performance with regard to resolution and retention time is given in Table 1. After conversion to capillary operation the instrument was fitted with a J&W DB-5 fused silica column (15 m) which is a somewhat more polar phenylmethylsiloxane bonded liquid phase. Consequently, the retention time of the piaselenol was increased, and this necessitated higher column temperatures to keep retention times within practical limits (Table 1). This system provided us with a good compromise of resolution and analysis time, and appeared to be free of any problems associated with interference from any neighboring peaks.

b) Results with the DB-5 capillary system: sample chromatograms and standard curves. A typical chromatogram is given in Figure 1. The piaselenol provides a fairly sharp peak at about 1.8 min with a variety of other peaks representing byproducts of reaction or the unreacted 4-nitro-o-phenylenediamine (NPDA) derivatizing reagent (2.4 min). The analysis time is somewhat limited by this large peak of unreacted material. However, the time (approximately 5 min) when run isothermally (Fig. 1) is consistent with the handling of large numbers of samples especially if autoinjection equipment is used. Presently, we are investigating the possibility of reducing the amount of NPDA to further minimize the effects of the presence of this peak. Preliminary results have indicated that reducing the concentration of this reagent by 50% has no measurable effect on the size of the piaselenol peak.

Figure 2 indicates the detector response is linear within

this range, and we estimate a working detection limit of 5 ng/L which has been adequate to evaluate all samples from softwater lakes thus far analyzed. We have tended to prefer peak height to area as a measure of concentration as this was somewhat less sensitive to interference by peaks generated by reaction byproducts. Precision of analyses for both standards and field samples on both peak height and peak area basis is provided (Table 2).

c) Effect of acid concentration on piaseleol chromatography. The most obvious effect of varying the piaseleol concentration is in the size of the NPDA peak which increases at low acid concentration (Fig. 3). However, high acid increases the size of the piaseleol blank, thereby increasing the effective detection limit. It is preferable to limit the magnitude of the blank, so the lowest possible acid concentration is recommended.

d) Analyses of environmental samples. Environmental samples from Lake 302 South of the Experimental Lakes Area presented a more complex sample matrix than the laboratory standards with an obvious increase in the number of peaks (Fig. 4). This increase in the number of peaks did not cause any obvious problems in data analysis, but may cause problems with other sample matrices. Data from Lake 302 South is plotted in terms of concentration (Fig. 5). Concentrations vary from 100-800 ng/L which is well above our detection limit of 5 ng/L, but at or below the limit of the conventional atomic absorption method. It is apparent that a hypolimnetic increase in Se^{IV} occurred during stratification. It is not clear, however, whether this is common in softwater lakes as we believe these are the first data of this kind produced. Concentrations in euphotic zone appear to be below 200 ng/L. These levels would likely be limiting to the growth of some phytoplankters based on our previously reported findings (Wehr and Brown 1985).

e) Continuing work. This project is continuing in the areas of Se speciation with particular emphasis on extending the assay to Se^{VI} and organoselenium which may exist in these waters. Also we need more information on sample storage and stability and any possible interfering compounds which may be encountered as we broaden the range of waters examined.

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Table 1. Comparison of selenium analysis with "Megabore" and capillary chromatography columns under various temperature programs.

Column	Column Pressure (lbs/in ²)	Initial temp. (oC)	Prog. rate (oC/min)	Final temp. (oC)	Se peak retention time (min)	Total analysis time (min)	Cooling time (min)	Relative resolution
Megabore DB-1	5.0	150	none	150	3.4	12.0	none	good
		170	none	170	1.6	9.5	none	fair
		190	none	190	1.1	8.0	none	poor
		200	none	200	0.8	6.5	none	poor
		120	25	190	2.8	10.0	2.5	good
Capillary DB-5	11.0	190	none	190	6.5	12.5	none	excellent
		230	none	230	3.1	6.8	none	excellent
		250	none	250	1.8	5.0	none	good*
		180	10	250	4.7	8.0	2.0	excellent

*recommended method

Table 2. Precision of injected samples (5 uL each) on DB-5 capillary column compared on the basis of peak height and area. Field data was from Lake 302 South, Experimental Lakes Area, Ont. (1985).

Sample	n	peak height		peak area			
		$\bar{x}$	SD	CV	$\bar{x}$	SD	CV
Standards							
100 ng Se ^{IV} /L	14	9333	513	5.5	628	82	13.0
250 ng Se ^{IV} /L	3	23673	60	0.2	1210	108	8.9
Lakewaters							
L 302-S; 8 Jun 8 m	5	17827	1496	8.4	1287	270	21.0
L 302-S 1 Aug 1 m	4	14460	1726	11.9	1105	234	21.2
L 302-S; 23 Oct 8 m	6	15777	656	4.2	1110	372	33.5

Fig. 1 Typical chromatogram for analytical standard (100 ng/L Se^{IV})

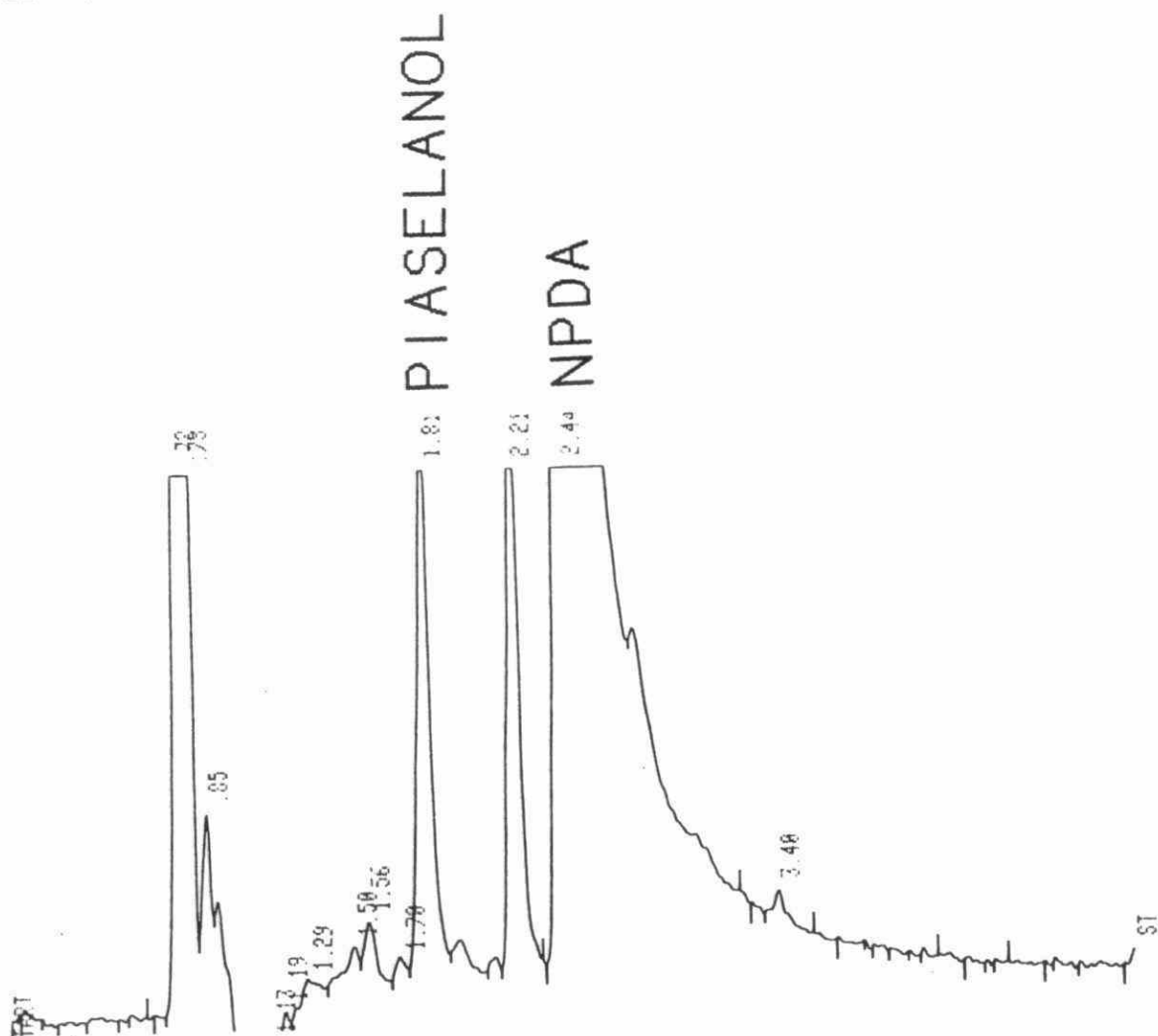


Fig. 2. Standard curve of piaseleenol derivative of Se^{IV} as measured by capillary chromatography (peak height basis)

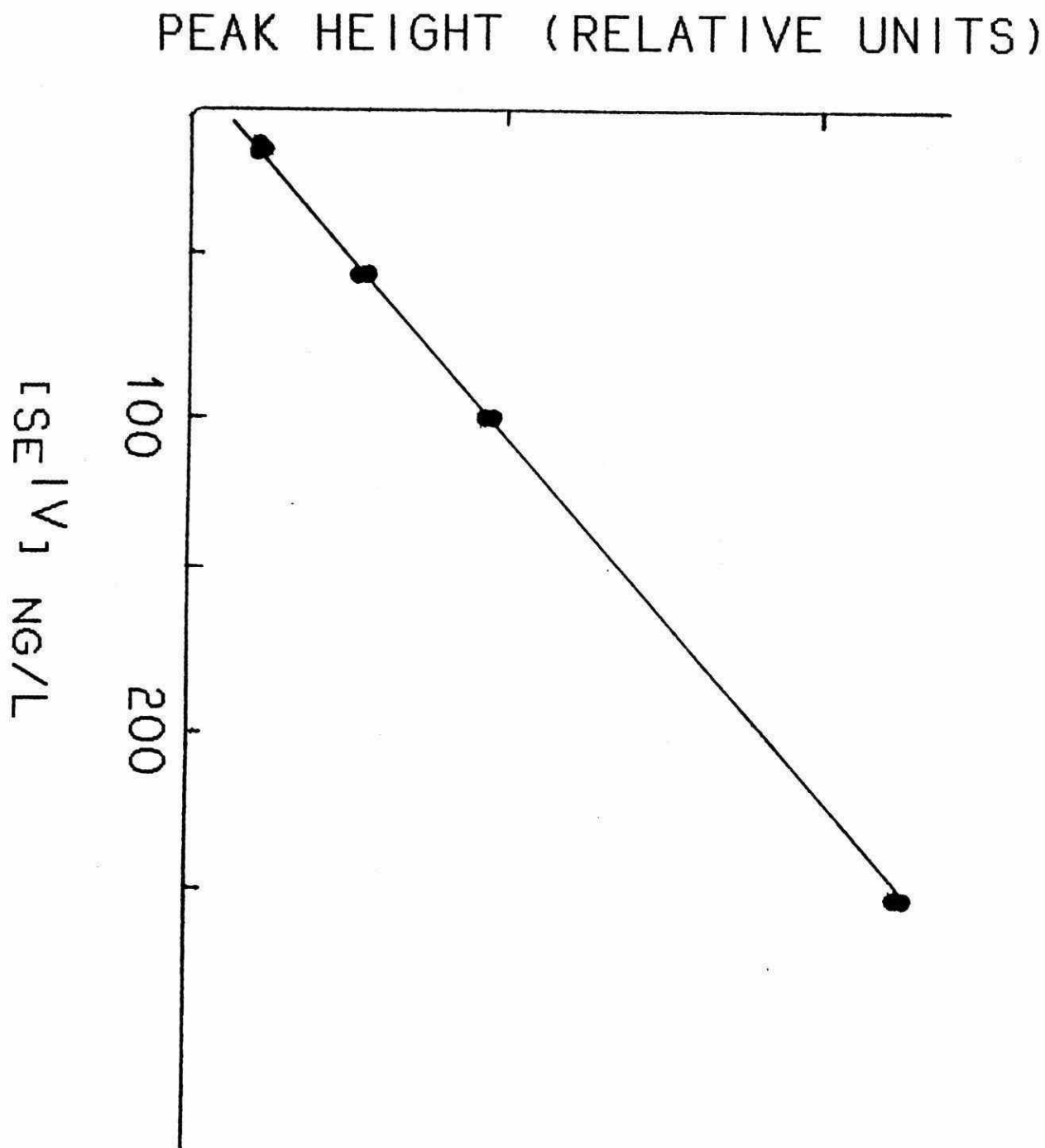


Fig. 3. Blank for standard curve at 0.01 M HCL (low acid)

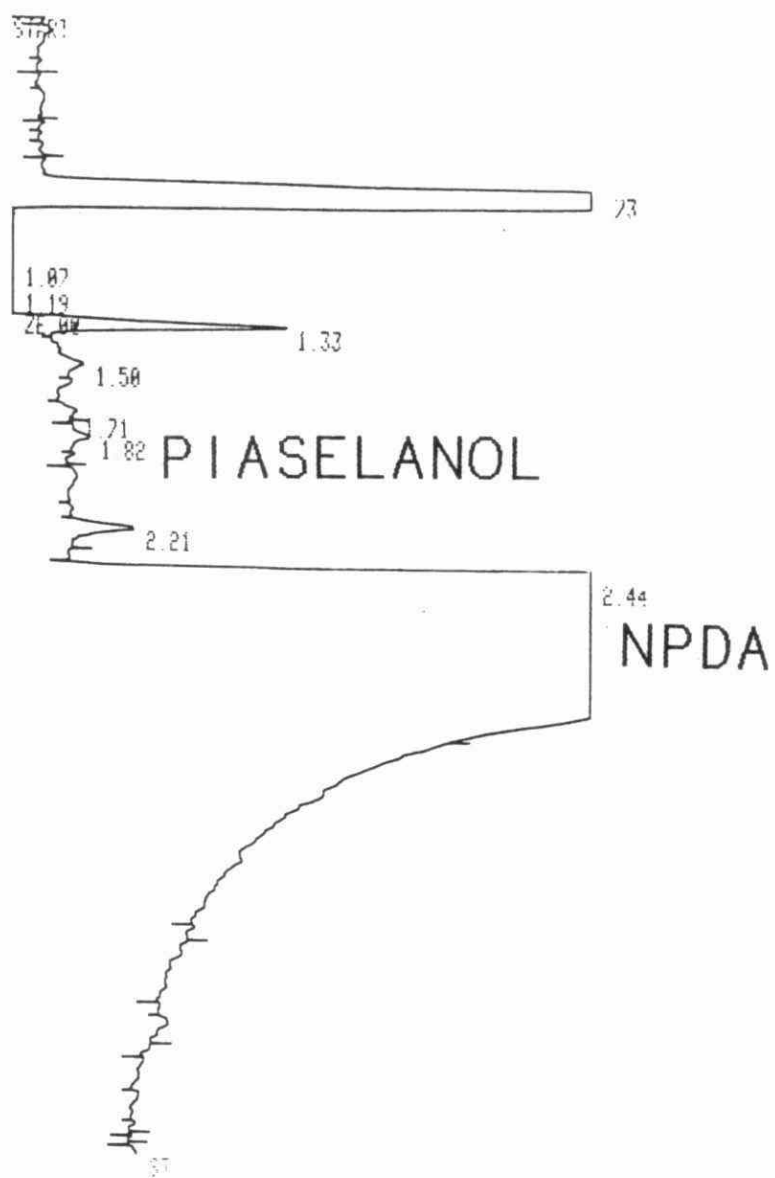


Fig. 4. Lake 302-S, Serv, Aug. 27, 1985, 1 m.

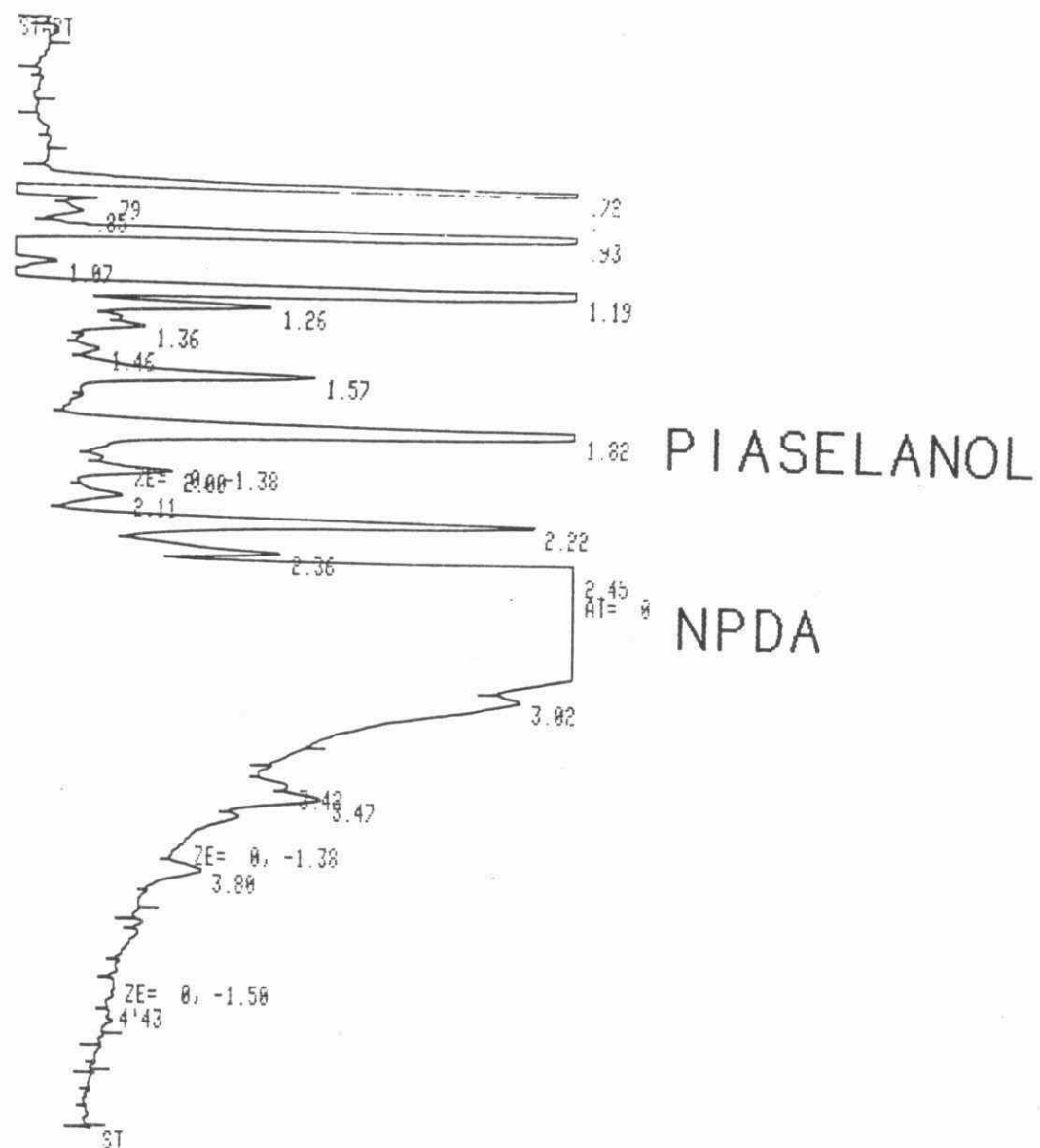
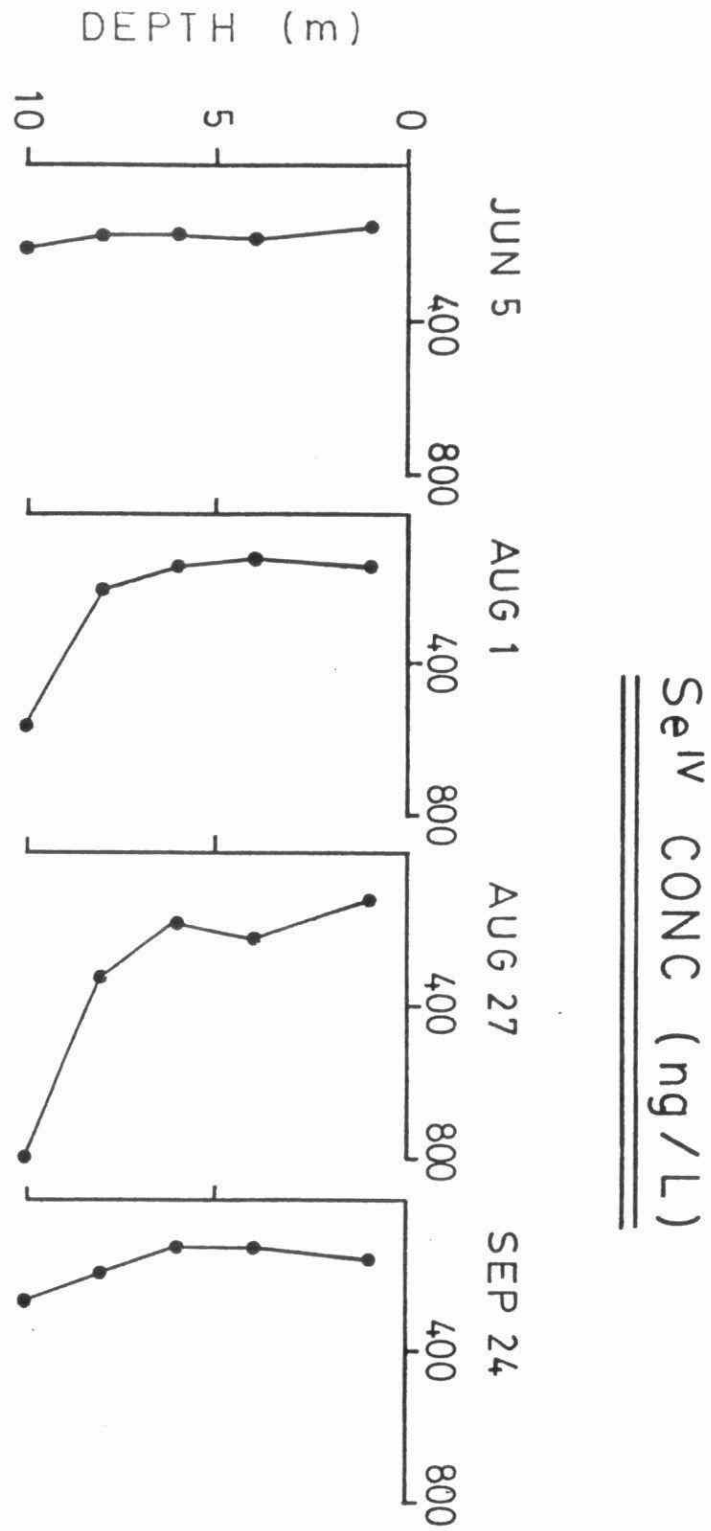


Fig. 5. Seasonal and depth variation of Se^{IV} in Lake 302-S as measured by the gas chromatographic method (1985).



EVALUATION AND OPTIMIZATION OF A TOC ANALYZER
USING UV - PERSULPHATE DIGESTION

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Paper not received at print time

DEVELOPMENT OF AN ULTRASONIC NEBULIZER FOR STABLE AND
REPRODUCTIVE PRODUCTION OF AEROSOLS FOR ATOMIC
SPECTROMETRIC ANALYSIS

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Ontario Ministry of the Environment

1. Introduction

The nebulization of samples is of central importance in atomic spectroscopic (AS) analysis. It has long been known that the conventional method of pneumatic nebulization is a far from efficient (6%) means of aerosol production for AS analysis [1]. Ultrasonic nebulization (USN), on the other hand has proven since 1964, to be a more efficient (86%) means of sample introduction [2].

Despite its seemingly superior efficiency, USN has yet to gain widespread acceptance. Many researchers however, report a variety of inadequacies with USN [3-9]. These include: 1) relatively poor short and long term stability, 2) longer clean-out times, 3) excessive long term drift, 4) memory effects and 6) high cost. Documented evidence shows [10-14] that continuous flow USN with desolvation offers a number of advantages over pneumatic nebulization as a means of sample introduction into the analytical ICP. The literature records many with descriptions of applications of USN to some of the most difficult analytical tasks encountered by the analyst [15-21]. Two recent commentaries however, have presented a more balanced view on the present status of USN. Barnes [21] has concluded that "full advantage of these nebulizers has not been taken by most ICP users. This is expected to change as the properties of the USN-ICP combination with practical samples are fully described." Thompson and Walsh [8] projected that the above deficiencies "could be improved upon by careful attention to details of design."

Early research in this laboratory has engendered a feeling of

optimism in the potential of USN. It is now believed that by proper redesign of the system and consideration of the limiting variables, such as stable and reliable transducer operation and 'proper' aerosol transport, a reliable and analytically useful USN could be developed.

2. Experimental

Schematic diagram of the USN design (as of date of writing manuscript).

Figure 1. Schematic diagram of ultrasonic nebulizer with desolvation.

Sample solution is pumped onto the water cooled transducer (a) with a peristaltic pump (Multistatic, peristaltic pump-HAAKE BUCHLER Instruments Inc.) via the sample inlet (b). The Ar carrier gas introduced through the drain tube (d) carries the aerosol through the heated tube (e) and condenser (f). The carrier gas plus sample is then directed into the plasma.

The power supply is a modified version of the BIONAIRE BT-200

ultrasonic humidifier (BIOTECH Electronics Ltd., Quebec, Canada). Specifications of modified and unmodified power supplies are given in Table 1.

To date, several condenser designs have been utilized or are under consideration. These include:

- 1) Freidrichs condenser
- 2) Cleisen condenser
- 3) Double surface Davies condenser
- 4) Modified Graham's condenser similar to that described by Olson et. al. [4]

Preparation of Fassel type [22] transducer assembly.

Using Kelvin's equation (1), and assuming that for aqueous solutions the product-quotient $\frac{8\pi\epsilon}{\rho} = 0.1515$, and for a driving frequency of 1.68 MHz, then λ is calculated to be 0.3772 cm. Therefore, the thickness of the quartz plate is given by $\phi = \lambda/2 = 0.1886$ cm. A small amount of bonding agent (Eccobond 55/Catalyst #11- W.R. Grace & Co., Burlington, Ont.) is placed between the transducer and the quartz plate. Bonding is effected by applying gentle pressure with the thumb and index finger, taking care that all air bubbles are excluded from the bond.

Tests were also done using polymers to coat the transducer working surface, instead of a quartz plate. Table 2 lists the polymers employed with the corresponding solvents.

The transducers were cleaned ultrasonically by immersion in an approximately 0.0007% detergent solution (Actionox, American Scientific Products, McGaw Park, IL) for 15 minutes, followed by rinsings in isopropyl alcohol and acetone, again ultrasonically for 15 minutes. Immediately prior to application of the polymer coating, the transducers were placed in a beaker saturated with isopropyl alcohol vapour and then allowed to air dry.

With the exception of polyurethane spray, all polymers were applied by dipping the transducer into a solution of the uncured or dissolved polymer. Different film thicknesses were obtained by varying the extent of dilution of the polymer solution.

The various coatings were evaluated based on the duration of adhesion of the coating to the transducer and, sensitivity and stability of the emission signal.

All calibration standards were prepared from serial dilutions of 1000 ppm stock solutions (BDH Chemical Co.). For ICP atomic emission analysis, eleven replicate, one second integrations were done per sample, per analyte.

3. Discussion

The power supply, as supplied, had an output frequency of 1.7 MHz, delivered at a 120 Hz ripple. The average frequency experienced by the transducer was therefore 1.24 ± 0.30 MHz. The addition of a capacitor filter in series with the circuit and then taking the output across the capacitor, resulted in improved

average output frequency and increased maximum power (see table 1).

As noted above, the Ag electrodes on the transducer are coated with approximately 1 μm of Ni metal, for protection. This type of Ag/Ni working surface leads to serious problems however, as the transducer was manufactured to operate using water and not acidic solutions. Nebulization of slightly acidic solutions ($> 1\%$ HNO_3) causes complete dissolution of the Ni and Ag in a matter of one hour or less.

To date, the method used to protect the Ag/Ni transducer working surface from degradation has been to bond a quartz plate over it. The thickness of the plate can be calculate from Kelvin's equation

(1)

$$\lambda = \left\{ \frac{8\pi\sigma}{\rho f^2} \right\}^{1/3}$$

where λ = wavelength (cm) of capillary waves

σ = surface tension (dyn/cm) of solution

ρ = liquid density (g/cm^3) of solution

f = driving frequency (MHz)

The quartz plate thickness is then given by

Bonding a quartz plate to the transducer results in several difficulties. Not only is the manual reproduction of the bonding process difficult, but for solutions of different sigma (σ) and rho (ρ), theoretically, the thickness of the quartz plate should also vary. Further, the thickness of the bonding glue layer effectively adds to the solid thickness that the ultrasonic waves

must traverse. If this thickness is not totally compatible according to Kelvin's equation, the waves are attenuated (and reflected back onto the transducer) and cause eventual burn-out of the transducer. As seen from table 3, using such a system, set up in this lab, a decrease in analytical performance was observed with time. Thus a new approach was investigated in this work for the protection of the Ag/Ni working surface. That is, to coat the surface with a polymer film.

To date, of the polymers investigated, only the polyurethane polymers and Aquasil showed any promise. All others dissolved within 15 minutes of initiating operation with 1% HNO_3 . One other polymer being considered is a combination of two polymers (D.E.R. 331 and D.E.R. 736, Dow Chemical Co., Sarnia, Ont.). This mixture has proven resistant to 2% HNO_3 , under operation, for a minimum of 4 hours. It has not however, been fully investigated. Table 4 gives a comparison of results obtained with pneumatic nebulization and ultrasonic nebulization utilizing Fassel's system, no coating and Aquasil coating on the transducer surface.

Production of aerosol at the transducer surface is a function of many parameters. The ones of primary importance are the liquid film thickness on the transducer surface, the viscosity of the solution (salt content), the applied oscillating frequency, the transducer temperature and the amplitude of the applied power. Even if one assumes that the viscosity, applied oscillation frequency and applied power remain constant, one cannot make the same assumption of the liquid film thickness on the transducer

surface or the transducer temperature.

The transducer temperature is directly dependant of the power applied, amount of solution coming into contact with the working surface and the cooling water flow under the transducer. (To a lesser extent also to the power reflected and the oscillating frequency). Assuming the applied power is maintained at an essentially constant level, then under optimum operating conditions, one can regulate the cooling water flow and other parameters to such an extent that the transducer temperature no longer is a variable in the production of aerosol.

However, the liquid film thickness on the working surface is still a problem. And, it is not one that can be readily or directly eliminated. But, there is an indirect method of compensation. This is, the automatic control of the applied power in response to variations in aerosol production efficiency due to variations in the surface liquid film thickness. A new power supply is being developed with a feedback system, set up to respond to differences in aerosol density passing a unit area at the inlet to the heated tube.

4. Results

As can be seen from plots I-IV, linear calibration curves were obtained for Co, Cu, Fe, Mg and Zn. Also, table 5 shows good agreement between accepted values and those obtained by the method proposed above, for the analysis of NBS sea water.

Table 6 gives a comparison of detection limits for the Meinhardt and Ultrasonic nebulization.

Table 1

Power supply specifications

Unmodified supply

Average output frequency	1.24 ± 0.3 MHz
Maximum power	50 W

Modified supply

Average output frequency	1.68 ± 0.01 MHz
Maximum power	75 W

Table 2

Polymers used and corresponding solvents or solvent mixtures

Polymer	Solvent(s)
PC 29, a polyurethane based circuit board coating (Dexter Hysol, Toronto, Ont.)	MEK
Polyurethane spray	---
Aquasil, a hydrophobic polymer (Chromatographic Specialties)	---
Polymethylmethacrylate	MEK toluene/acetone
Polystyrene (Aldrich Chemical Co.)	MEK
Polyethylene	toluene/acetone
Silicone rubber	n-heptane

Table 3

Sensitivity as a function of time with Fassel's system

<u>Cu sensitivity*</u> (ppb)	<u>Time (hrs)</u> <u>of operation</u>	<u>RSD (%) for 2 ppm Cu</u>
16	0	3.5
21	2.5	3.7
29	5.5	3.3

* Concentration required for 0.0044 absorbance

Table 4

Comparison of results for various systems

<u>Type of system</u>	<u>Cu sensitivity</u> <u>(ppb)</u>	<u>RSD</u>
(1) pneumatic	87	0.8
(2) Ultrasonic (no coating)	15	1.8
(3) Ultrasonic (Aquasil)	11	0.6
(4) Ultrasonic (Fassel [25])	16	3.5

Table 5

Comparison of experimental and accepted values for the analysis of NBS sea water.

Concentration ($\mu\text{g/L}$)

<u>Element</u>	<u>this method</u>	<u>NBS certified</u>	<u>RSD(%)</u>
Co	28.6	26 ± 1	1.4
Cu	24.7	$21.9 \pm .4$	2.1
Fe	98.9	99 ± 8	1.2
Zn	71.6	66 ± 2	3.1

Table 6

Comparison of limits of detection

<u>Element</u>	<u>LOD ($\mu\text{g/L}$)</u>	
	<u>Meinhardt</u>	<u>USN*</u>
Co	3.4	0.70
Cu	2.3	1.07
Fe	1.2	0.31
Mg	13.7	2.40
Zn	4.0	0.88

* With desolvation

Table 7

Ultrasonic nebulizer operating parameters

Sample flow rate	6 mL/min
Ar carrier gas flow rate	0.95 L/min
Max. forward power	75 W
Oscillating frequency	1.68 MHz

Table 8

Transducer Specifications

Dielectric	Bi/Tc
Load.....	50 Ohms
Operating Frequency	1.68 MHz
Electrodes	Ag
Protective coating	Ni

Table 9

Heated tube specifications

Quartz	
Length	12 cm
Inner diameter	10 mm
Electrothermal heating tape	100 W (Canlab)
Temperature	80 - 200 °C

Table 10

ICP operating parameters

Model	ARL 34000 ICPAQ
Incident power	1600 W
Reflected power	< 3 W
Plasma gas	0.8 L/min
Coolant gas	12 L/min
Observation height in coil	15 mm

Table 11

Analyte lines used

<u>Element</u>	<u>Wavelength (nm)</u>	<u>Order</u>
Co	228.62	2
Cu	324.75	2
Fe	259.94	2
Mg	279.08	2
Zn	213.86	3

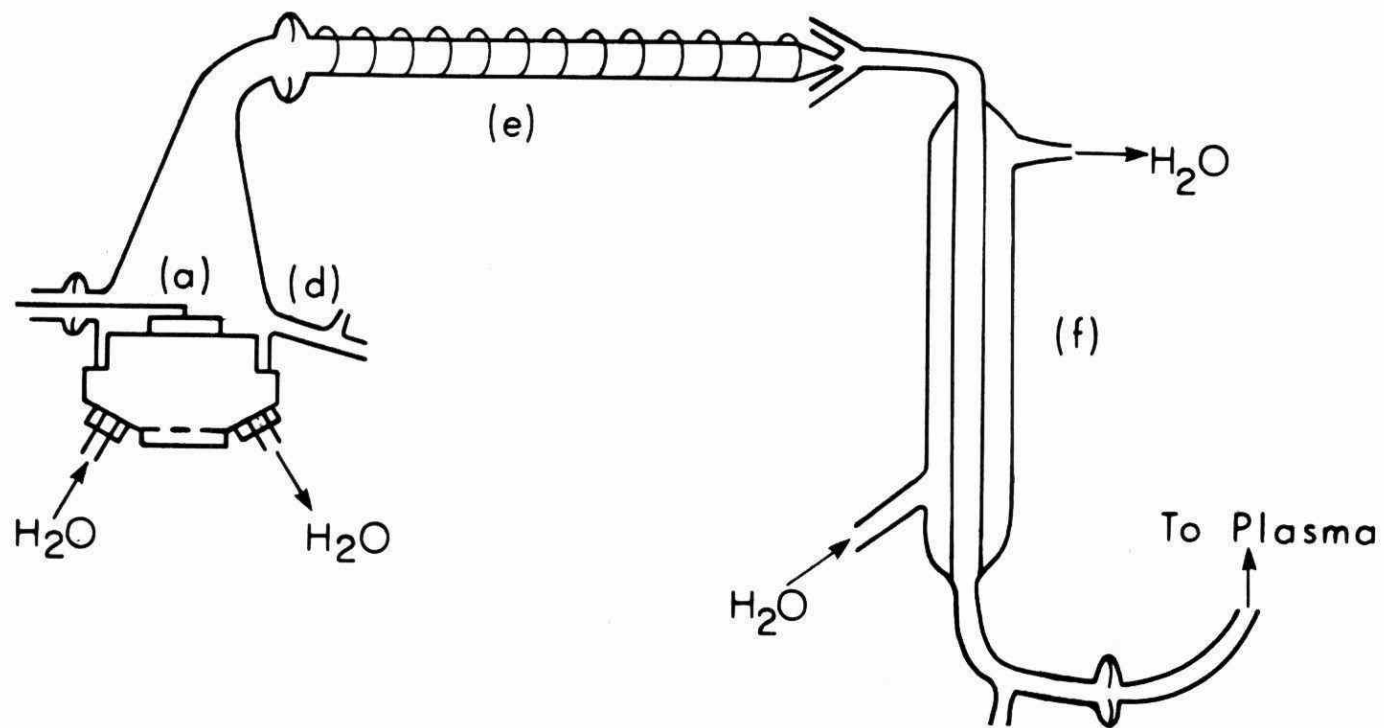


FIGURE 1

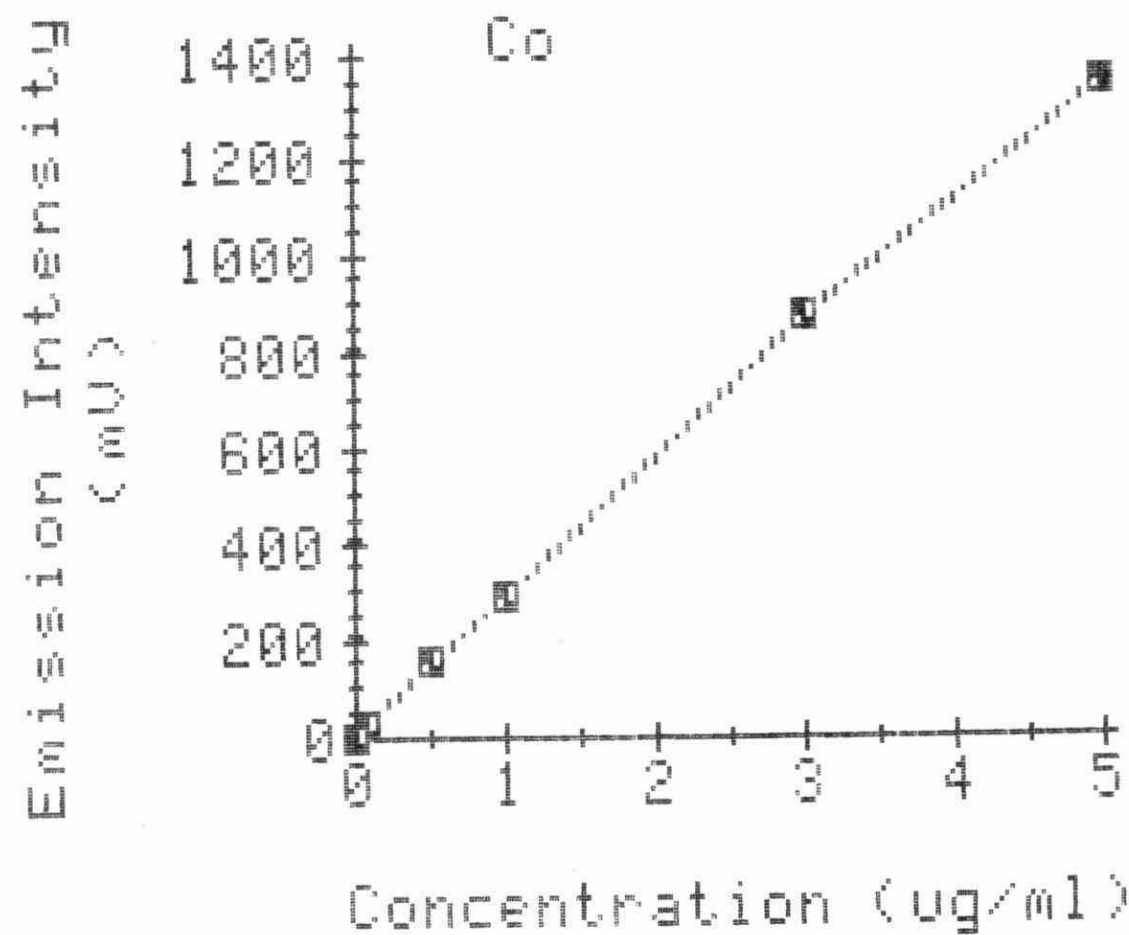


Figure 2: Calibration plot for Co.

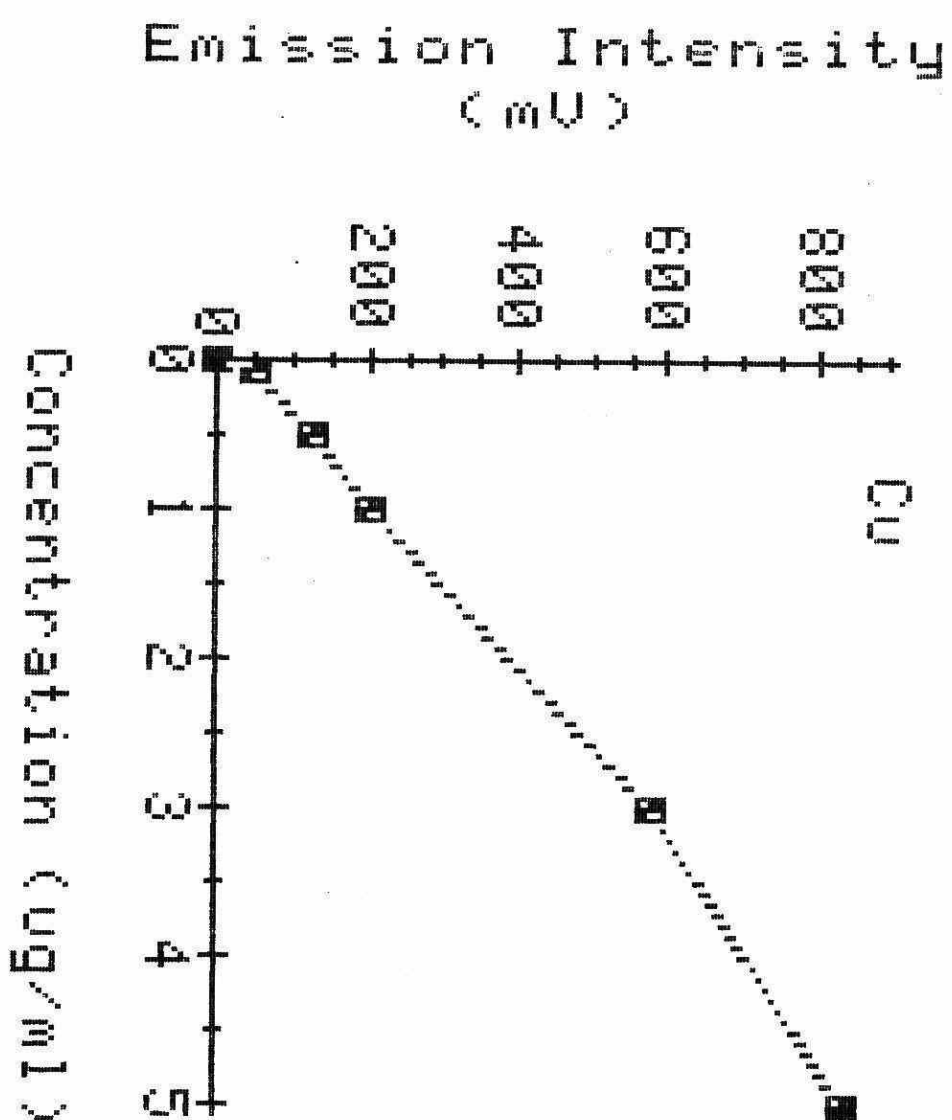


Figure 3: Calibration plot for Cu.

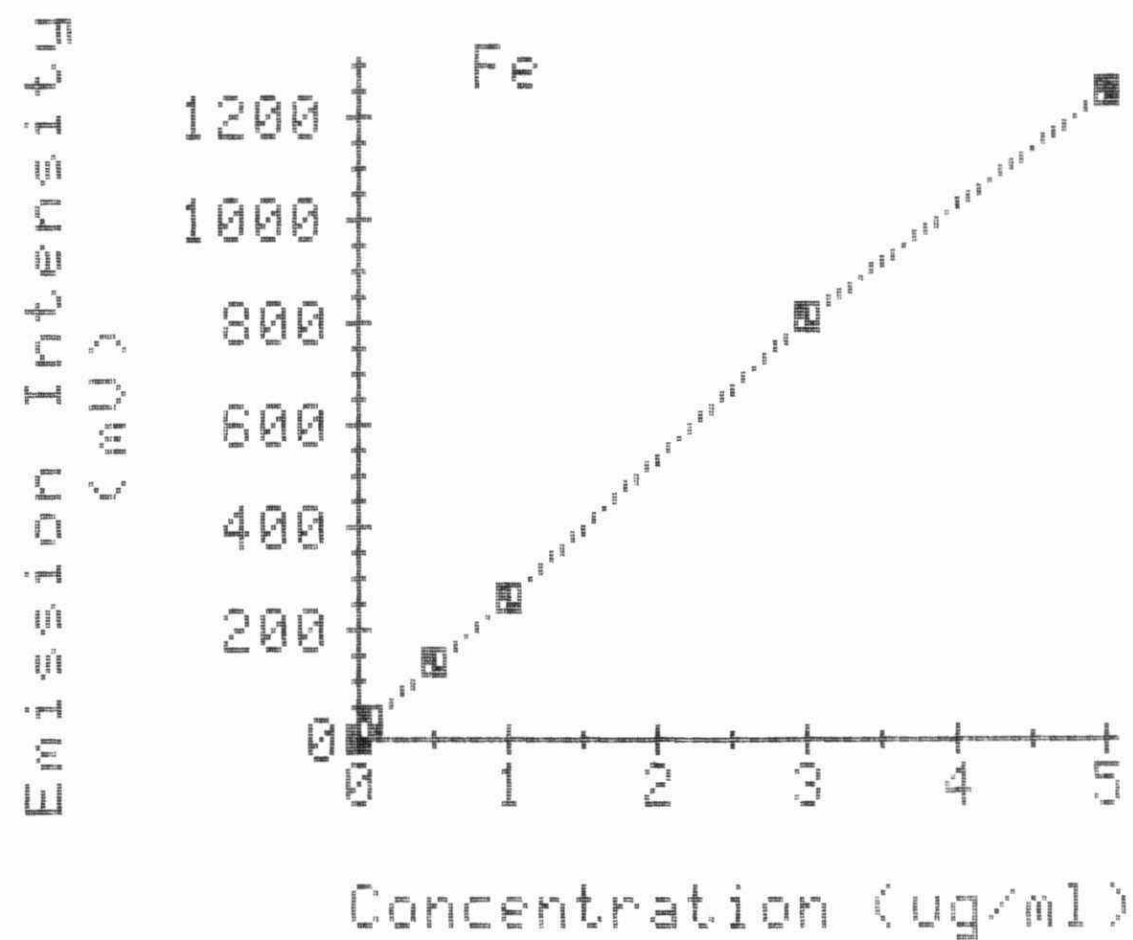


Figure 4: Calibration plot of Fe.

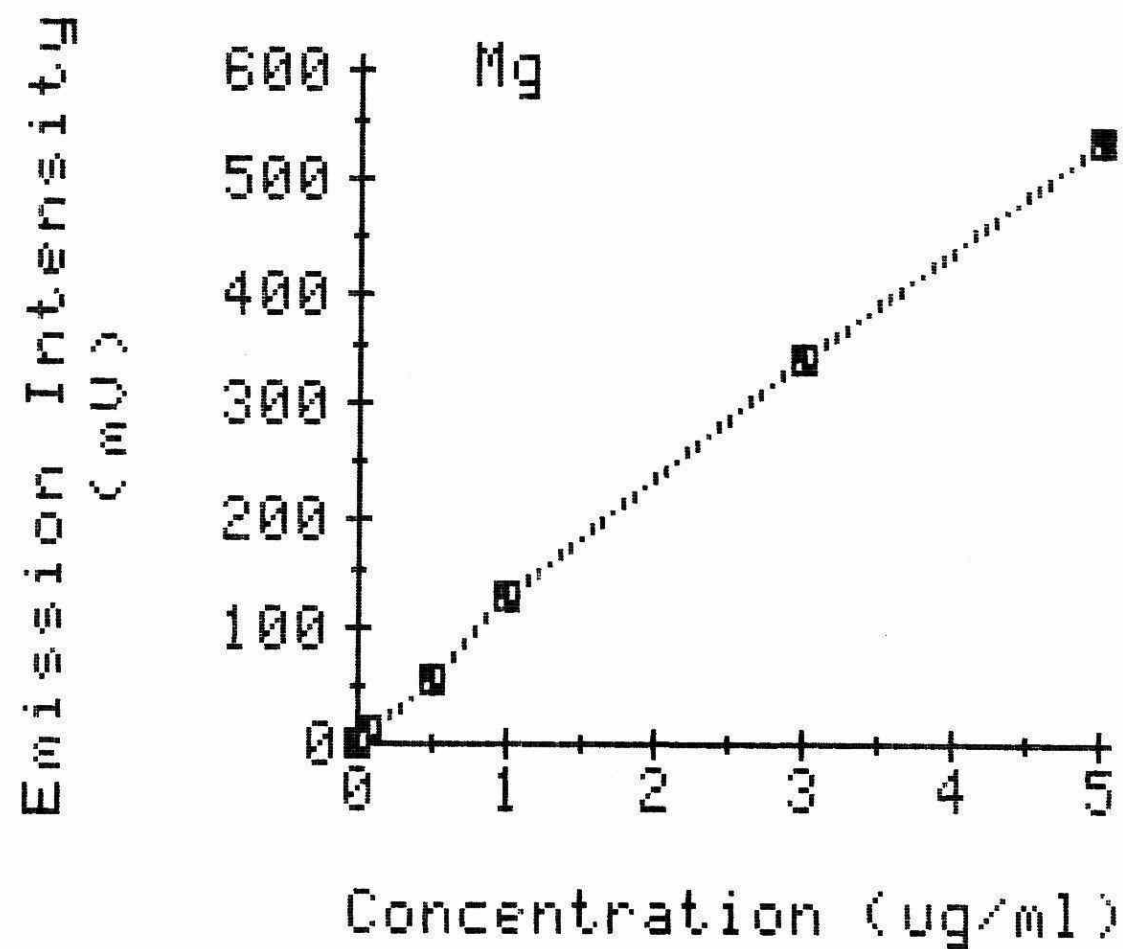


Figure 5: Calibration plot for Mg.

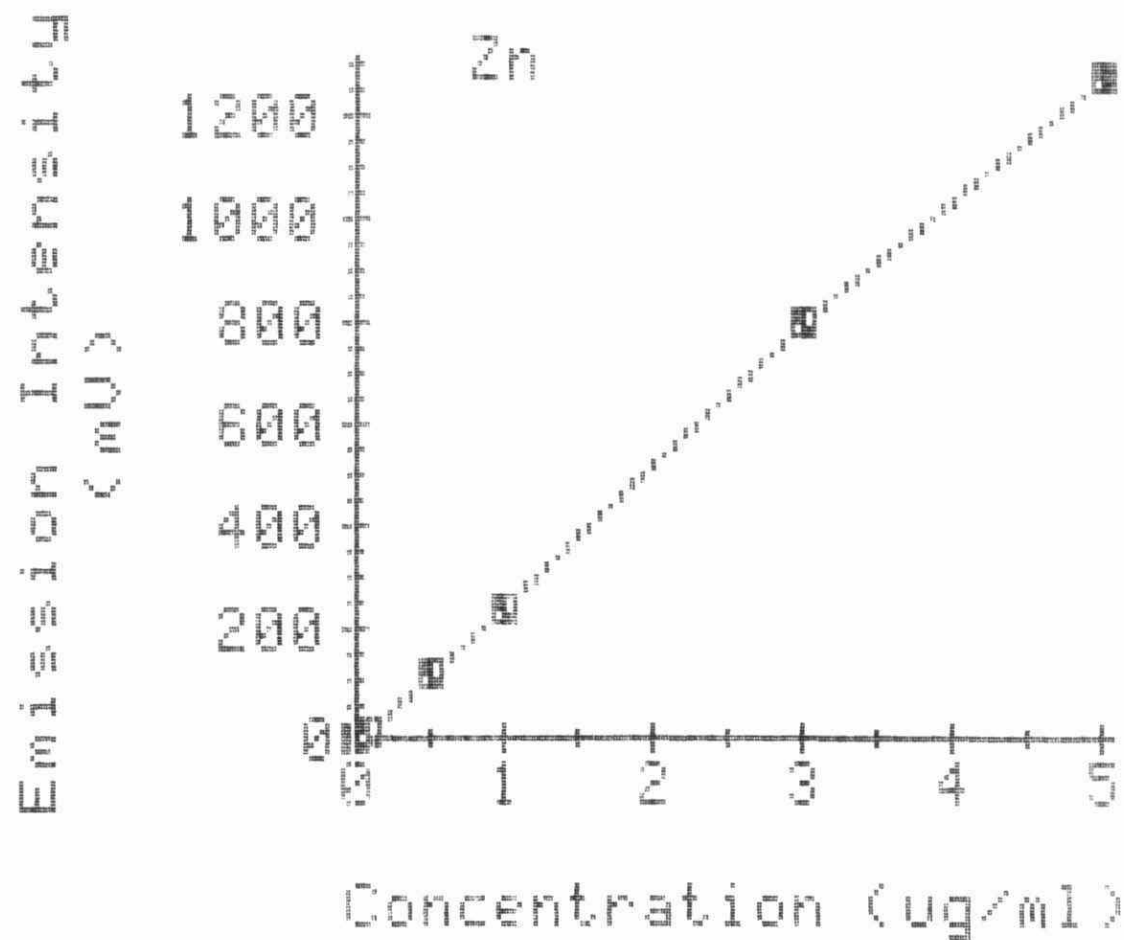


Figure 6: Calibration plot for Zn.

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Development of Recombinant DNA Probes to Determine the Origin of
Fecal Streptococci at the Toronto Area Beaches

W.C. Bradbury, M.A. Marko, L. Papageorgiou, D. Leicht and D. Rego

Abstract

Total cellular DNA from fecal Streptococci from goose, human and gull origin were digested with restriction endonucleases. The digestion patterns were grouped into families by visual inspection for commonality. An isolate from goose feces was chosen as a candidate for preparing the goose probe because this "family" included both sediment and fecal isolates.

The total cellular DNA was digested with the restriction endonuclease Bam HI and inserted into the Bam HI site of the cloning vector pUC 9. The colonies of E. coli which had been transformed by a recombinant plasmid were screened for plasmid content. The plasmids were digested with Bam HI and electrophoresed in agarose gels. The DNA was transferred to millipore membranes (Southern blots) and probed with 32-P-labeled total cellular DNA from Streptococcus faecalis var liquefaciens of human origin.

An insert of 6.6 Kb which did not hybridize to the probe was chosen as a candidate for the goose streptococcal DNA probe. This plasmid was radiolabeled with 32-P and used to analyze the total cellular DNA of fecal Streptococci of goose, human and gull origin.

The probe hybridized to the "family" of goose fecal DNA from which it was derived and not to the human fecal streptococcal DNA of non-laboratory workers. In addition only one the twenty (1/20) gull isolates reacted positively.

In a similar manner a probe for human fecal streptococcal DNA was generated from Streptococcus faecalis var liquefaciens of human origin and candidate recombinant plasmids were selected by their inability to hybridize with the total cellular DNA of Streptococcus var liquefaciens of gull origin. A plasmid with an insert of 2.0 Kb was found to react with four of twenty (4/20) fecal streptococcal DNA of human origin but not with any of goose or gull origin.

Introduction

The recent increase in the closing of Toronto's public beaches has been in response to high fecal coliform and fecal streptococcal counts. Although these assays are useful indicators of the presence of water-borne pathogens, they provide little information on the source of fecal contamination. Based on the fact that Fecal Coliform:Fecal Streptococci Ratios (FC:FS) vary between human and animal feces, an attempt has been made to use this assay to trace sources of fecal contamination (1,2,6). However, it has been shown that these ratios are highly

unreliable due to the different survival capacities of fecal coliforms and fecal Streptococci in differing, especially urban water environments.

Furthermore, the close association between humans, gulls, geese and other animals along the Toronto waterfront has shown that there is a high degree of similarity between intestinal flora of these organisms. A cumulation of these problems has emphasized the fact, that at present there is no reliable method to trace the source of the fecal contamination.

Studies at the DNA specific level exploiting restriction endonuclease analysis (REA) and DNA-DNA hybridization techniques may be the key to defining streptococcal organisms that are host specific. In this study we have attempted to identify specific DNA probes that can distinguish between human and non-human fecal contamination. Such specific DNA probes can be used to assess the contribution of each source of fecal contamination along the Toronto waterfront.

Materials and Methods

Bacteria. Isolations were performed according to the standard bacteriological methods recommended for these organisms (6). These organisms were identified by the API system and included fecal isolates from humans and gulls (Table 1 and 2).

Chromosomal DNA preparation. Streptococcal chromosomal DNA was prepared as previously described (3).

Restriction endonuclease digestion of DNA. Approximately 1.0 to 2.0 ug of streptococcal DNA sample was used for each restriction endonuclease digest. The DNA was digested according to the supplier's specifications (Boehringer-Mannheim, St. Laurent, Quebec) except 10 to 20 U of enzyme were used per ug of DNA.

Gel electrophoresis and photography. Sample preparation, the conditions of agarose gel electrophoresis and photography of the resultant restriction patterns were performed as previously described (3).

Cloning procedures. Streptococcal chromosomal DNA was purified and cloned into a pUC-9 plasmid vector according to the method previously described (9,10).

Preparation of 32-P-labeled DNA probes. Duplex DNA samples were labeled with radioactive deoxynucleotide triphosphates by nick translation as previously described (4). The specific activities

obtained were routinely 10^8 cpm/ug of DNA. The labeled probes were used within one month of preparation.

Southern transfer and hybridization to 32-P-labeled probe DNA. Southern (8) blotting experiments were performed according to methods previously described (4).

Results

Total cellular DNA from fecal Streptococci from goose, human and gull origin were digested with restriction endonucleases. The digestion patterns were previously grouped into families by visual inspection for commonality (15). An isolate from goose feces identified as Streptococcus durans (#181 strain) was chosen as a candidate for cloning procedures in preparation for its use as a goose probe because this "family" included both sediment and fecal isolates (15).

The total cellular DNA was digested with the restriction endonuclease Bam HI and inserted into the Bam HI site of the cloning vector pUC 9. The colonies of E. coli which had been transformed by a recombinant plasmid were screened for plasmid content. The plasmids were digested with Bam HI and electrophoresed in agarose gels (Figure 1a, 1b and 1d). The DNA was Southern blotted to millipore membranes and probed with 32-P-labeled total cellular DNA from Streptococcus faecalis var liquefaciens of human or gull origin (Figure 1c and 1e).

Thus candidate recombinant plasmids were selected on the basis of their inability to hybridize with the total cellular DNA obtained from human (Figure 1c) and gull (Figure 1e) sources.

An insert of 6.6 Kb (Figure 1a) which did not hybridize to the human or gull probes was chosen as a candidate for the goose streptococcal DNA probe, herein referred to as goose probe #807 (Figure 1c, 1e).

Probe #807 was next radiolabeled with 32P and used to analyze the total cellular DNA of fecal Streptococci of goose, human and gull origin (Figure 2).

The probe hybridized to the "family" of goose fecal streptococcal DNA from which it was derived (Lane 7 in Figure 2a, 2b, 2c and 2d) and not to the human fecal streptococcal DNA of non-laboratory workers (data not shown). However probe #807 reacted with human fecal streptococcal DNA of laboratory workers (Figure 2b and 2d) which suggested the the workers were colonized by the fecal streptococcal strains that they handled in the laboratory. Furthermore probe #807 reacted with only one of eight (1/8) goose restriction families identified in our earlier studies (15) which suggested a high degree of specificity. In addition only one of the twenty (1/20) gull isolates reacted positively (Figure 3).

In a similar manner a probe for human fecal streptococcal DNA was generated from Streptococcus faecalis var liquefaciens of human origin and candidate recombinant plasmids were selected by their inability to hybridize with the total cellular DNA of Streptococcus var liquefaciens of gull origin. A plasmid with an insert of 2.0 Kb was found to react with four of twenty (4/20) fecal streptococcal DNA of laboratory workers (Figure 4b) but not

with any of goose or gull origin nor with twenty non-laboratory workers (data not shown).

Discussion

This study extends our earlier observations as to the usefulness of REA or gene fingerprinting techniques for determining the sources and routes of transmission of microbial organisms. In particular we have utilized these techniques for tracing infections (3,5,7,13) and pollution sources (15). Combining cloning and DNA-DNA hybridization techniques we have developed a method whereby unique DNA sequences can be identified that would serve as specific probes for tracing and enumerating the pollution sources in waterways. In particular, we have developed a goose-specific probe which can now be used to enumerate the contribution of goose feces to the over-all bacterial contamination at the Toronto waterfront. In addition we compared results obtained with the goose probe with those obtained from a cloned-fragment probe derived from human streptococci and a gull whole-cell DNA probe.

The results of this study indicate firstly that the goose probe #807 was very specific and did not react with streptococcal strains from non-laboratory workers nor from gulls, but did react significantly with streptococcal strains obtained from laboratory workers. Interestingly, the laboratory workers were involved in the field collections and the testing of the fecal and environmental samples of Streptococci. Thus probe #807 specifically identified a problem associated with this approach, in that lab workers became colonized with the various streptococcal strains handled. On the other hand further tests with the goose probe using streptococcal strains obtained from non-lab workers indicated a complete lack of reaction. From an epidemiological and health point of view these results suggest that fecal streptococcal strains from the waterfront can colonize humans. From a microbiological point of view such human sources should be avoided if attempting to establish types of specific bacteria associated with humans. Thus the probes are only as good as the selection process used to determine a host's microbial flora - "garbage in garbage out".

Secondly, the specificity of DNA probes is crucial and it was for this reason that we initially chose a streptococcal strain that was not the most common strain found in association with goose feces. From this isolate, identified biochemically as a S. durans (strain #181) we obtained the unique DNA fragment for the goose probe #807. If, on the other hand, had we chosen the most common strain found in goose feces as the candidate for development of the probe, a highly specific DNA fragment may not have been elucidated. Based on the suggestion that human sewage is a significant if not the biggest contributor to the overall bacterial pollution at the Toronto waterfront then the most common isolates found in association with either goose or gull hosts could conceivably be of human origin. Thus we sought a less

common or more host-specific strain for the development of probe #807.

Thirdly, the technique of genome fingerprinting, combining REA, cloning methods and nucleic acid hybridization techniques, as demonstrated herein, can be used effectively to define the sources as well as the routes of transmission of microbial flora. Presently used conventional techniques based on phenotypic characteristics are unreliable and cannot discriminate strains at the subspecies level. This is clearly seen by comparing Tables 1 and 2 with the genome fingerprints. In a number of cases strains with identical or indistinguishable fingerprints have been identified by convention biochemical techniques as being different species or subspecies. By comparison the capability of REA and in particular the discriminating power of gene probes to identify minute differences or Restriction Fragment Length Polymorphisms (RFLP's), among otherwise "identical" strains, is remarkable.

The discrepancies between phenotypic-based microbial identification systems and the more novel genetic-based techniques as developed herein is a serious problem in the establishment of useful fecal markers for pollution tracing. Thus our results suggest that a critical examination of the taxonomic relationship among the Group D fecal Streptococci must now be undertaken particularly with respect to their host range, if these organisms are to be used as reliable markers of fecal contamination and standards for water quality assurance. Homology studies should be performed on this large group of organisms in order to establish the most important and unique phenotypic characters that can simply be used to discriminate the various strains.

Similarly, specific fragments of DNA used in these probes can easily be made into expression vectors the protein products of which may be used to developed specific monoclonal antibody probes. Such immunologic-based probes may be more easily adapted to rapid ELISA systems for use at the day-to-day microbiology bench. Our studies are continuing in these directions.

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FIGURE LEGENDS

FIGURE 1:

Selection of recombinant DNA probes for fecal Streptococci isolated from geese.

1a. Bam HI digests of pUC 9 recombinant #807 with lambda DNA Hind III digest markers in kilo base pairs (kbp).

1b. Bam HI digests of pUC 9 recombinants of goose fecal Streptococcus durans #181 DNA.

1c. Autoradiograph of the DNA transfer (blot) to a millipore membrane of the agarose gel seen in figure 1b, probed with 32-P-labeled total streptococcal DNA from a human source. The inserts in lanes 1 and 2 (cf black arrows with white arrows in figure 1b) reacted with the probe whereas the fragment in #807 did not.

1d. Additional BamHI digests of pUC 9 recombinants of goose fecal Streptococcal durans #181 DNA (including #807).

1e. Autoradiograph of the DNA blotted from the gel seen in figure 1d and probed with streptococcal DNA derived from a gull source. The inserts indicated by black and white arrows indicate reactions with the probe.

FIGURE 2:

Reaction of the goose probe #807 with streptococcal DNA from humans (i.e. laboratory workers), goose, gull and water-sediment sources.

2a. Bam HI digests of streptococcal DNA. Lanes 1 - 6 contain human source (i.e. laboratory workers) streptococcal DNA; Lane 7 contains goose Streptococcal durans #181 DNA; Lanes 8 and 9 contain sediment source streptococcal DNA; Lane 10 contains gull source streptococcal DNA.

2b. Autoradiograph of DNA blot from figure 2a probed with goose probe #807 (black arrow indicates the single Bam HI fragment reaction obtained with this probe).

2c. Cfo I digest of the same source DNA as in figure 2a.

2d. Autoradiograph of the 2c DNA blot, probed with #807. Restriction Fragment Length Polymorphisms (RFLP's) are evident in Lanes 5 and 6.

FIGURE 3:

Reaction of the goose probe #807 with streptococcal DNA from gull sources.

3a & 3c. Bam HI digests of streptococcal DNA from gull sources.

3b. Autoradiograph of DNA blotted from 3a and probed with probe #807. Only Lane 1 reacts (black arrow).

FIGURE 4.

Reaction of human DNA probe #1126 with streptococcal DNA from human sources

4a & 4c. Bam HI digests of streptococcal DNA from human sources:

4a. Lanes 1 - 6 = Restriction Family A (see Tables 1 and 2)
Lanes 7 - 8 = Family B
Lanes 9 - 10 = Family C

4c. Lanes 1 - 4 = Family D
Lanes 5 - 6 = Family E
Lanes 7 - 8 = miscellaneous isolates

4b. Autoradiograph of the DNA blot of Figure 4c probes with #1126. Reactions are clearly evident in Lanes 1 - 4.

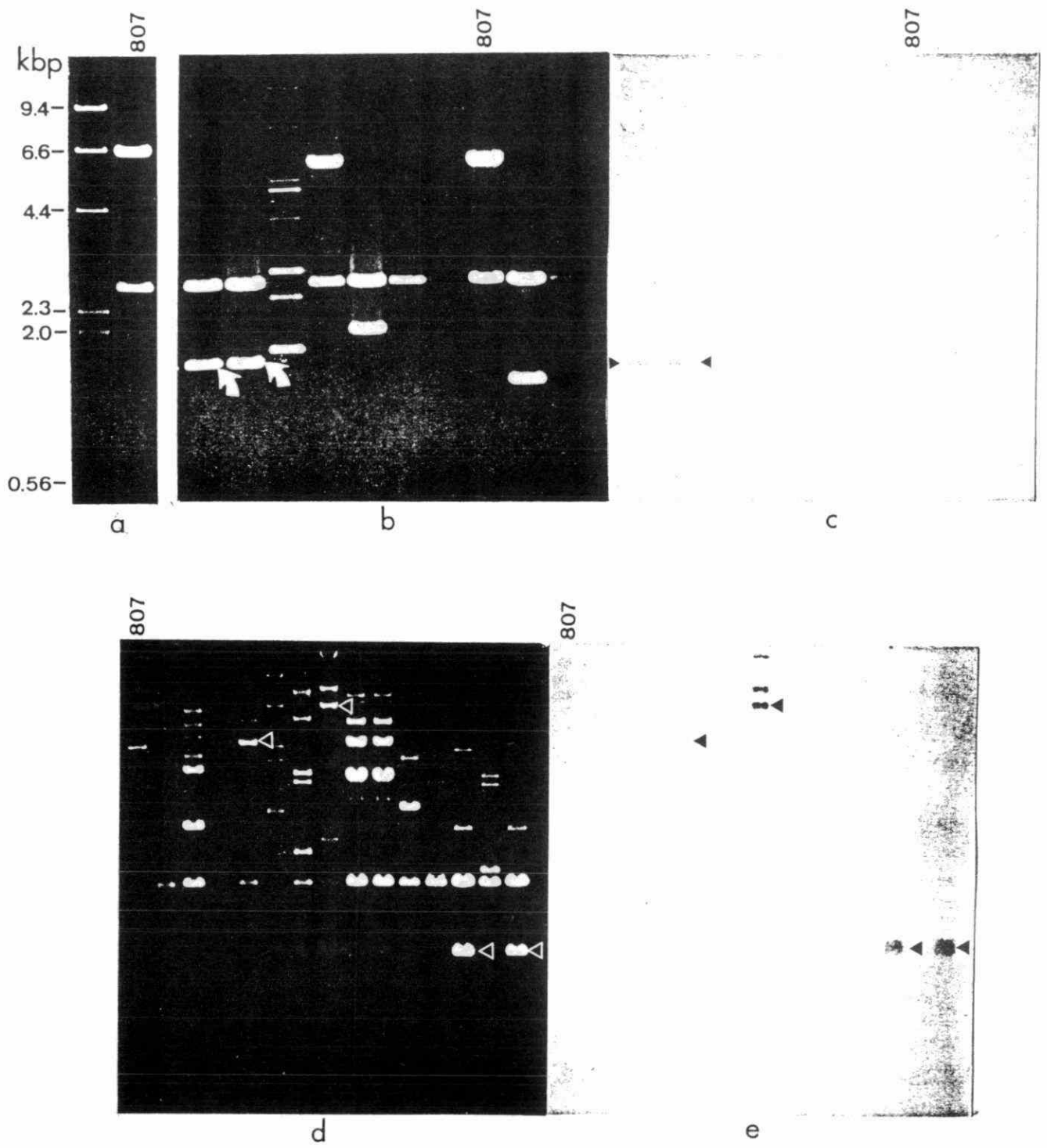


FIG. 1

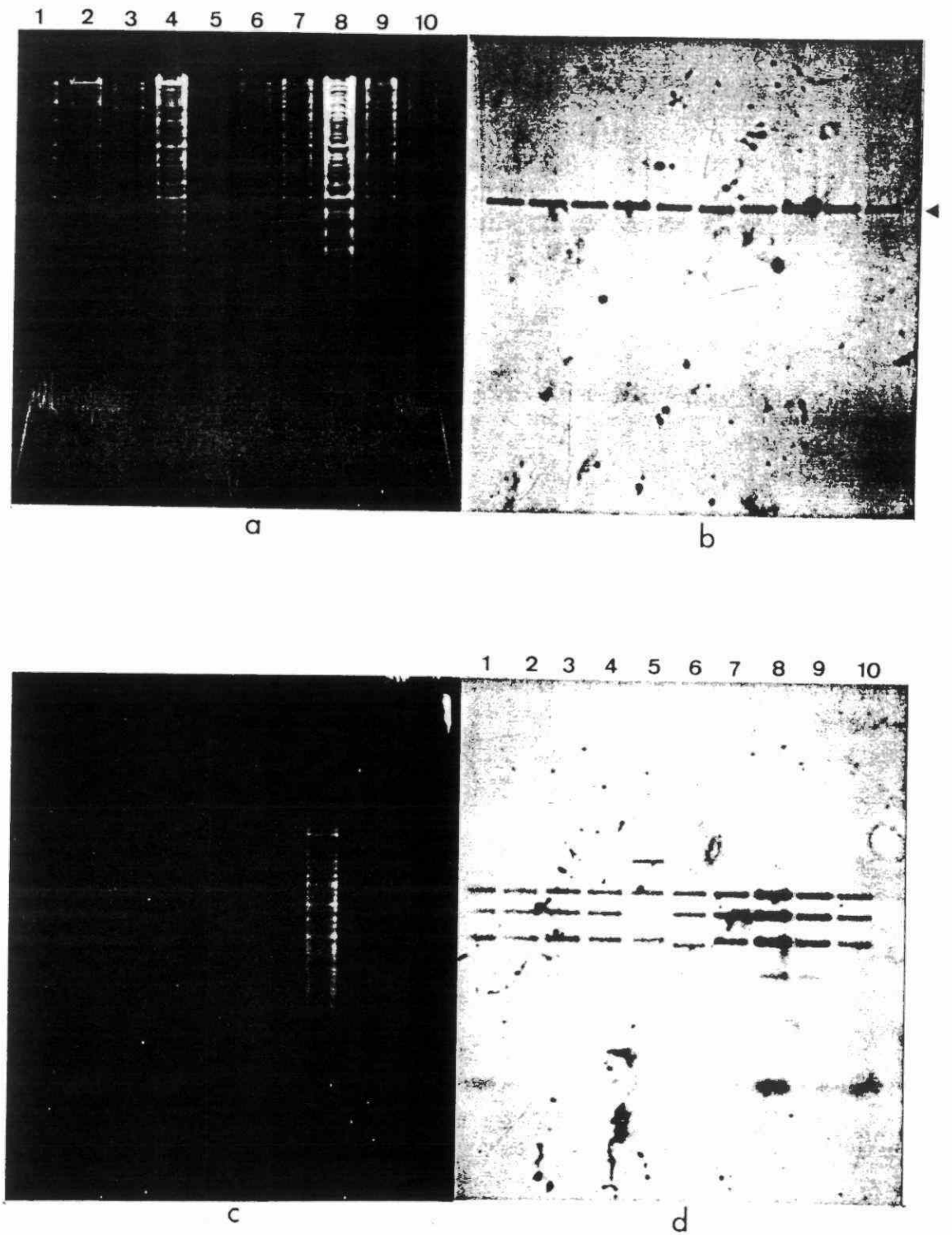


FIG.2

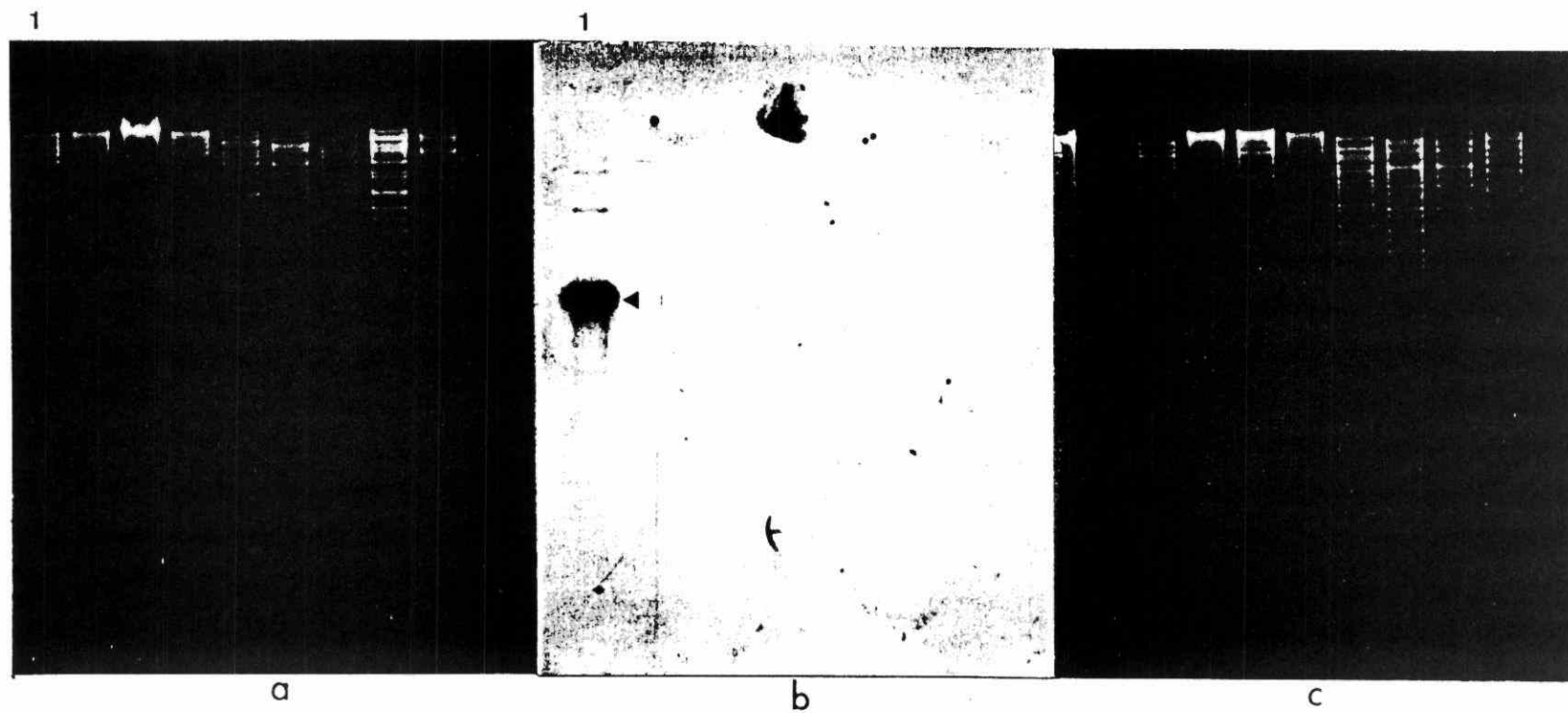


FIG.3

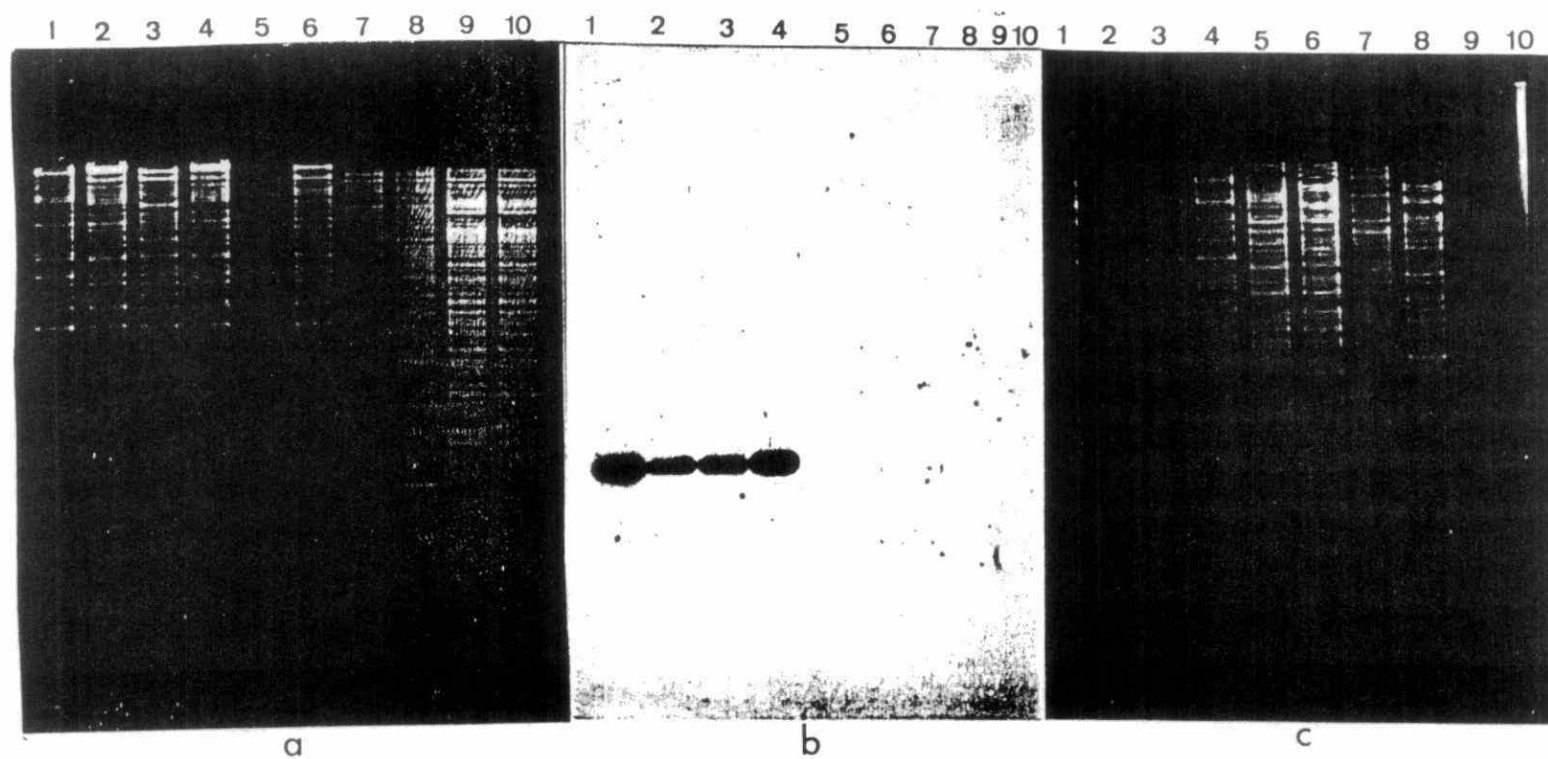


FIG.4

Table I

Biochemical identity and restriction endonuclease (REA) families of human source Streptococci and their reactions with goose probe #807 and human probe #1126.

<u>Identity</u>		#807	<u>PROBE</u> #1126	REA Family
1.	<u>S. durans</u> (Fig.4 c, lane 1)		+	D
2.	<u>S. durans</u> (Fig.4a, lane 1)	+		A
3.	<u>S. durans</u> (Fig.4a, lane 7)			B
4.	<u>S. durans</u> (Fig.4a, lane 2)	+		A
5.	<u>S. durans</u> (Fig.4a, lane 3)	+		A
6.	<u>S. durans</u> (Fig.4a, lane 4)	+		A
7.	<u>S. durans</u> (Fig.4a, lane 8)			B
8.	<u>S. faecium</u> (Fig.4a, lane 9)			C
9.	<u>S. faecium</u> (Fig.4a, lane 10)			C
10.	<u>S. faecium</u> (Fig.4c, lane 5)			E
11.	<u>S. faecium</u> (Fig.4c, lane 6)			E
12.	<u>S. faecium</u> (Fig.4c, lane 7)			
13.	<u>S. faecium</u> (Fig.4c, lane 8)			
14.	<u>S. faecalis</u> var. <u>liquefaciens</u> (Fig.4c, lane 2)		+	D
15.	<u>S. faecalis</u> var. <u>liquefaciens</u> (Fig.4c, lane 3)		+	D
16.	<u>S. durans</u> (Fig.4a, lane 5)	+		A
17.	<u>S. faecalis</u> var. <u>liquefaciens</u> (Fig.4c, lane 4)		+	D
18.	<u>S. faecalis</u> var. <u>liquefaciens</u> (Fig.4c, lane 9)			
19.	<u>S. faecalis</u> var. <u>liquefaciens</u> (Fig.4c, lane 10)			
20.	<u>S. faecalis</u> var. <u>liquefaciens</u> (Fig.4a, lane 6)	+		A

Table 2

Biochemical identity and restriction endonuclease (REA) families of gull source Streptococci and their reactions with goose probe #807 and human probe #1126.

Identity	#807	PROBE #1126	REA Family
1. <u>S. faecalis</u> var. <u>liquefaciens</u>	+		A
2. <u>S. faecalis</u> var. <u>casseliflavus</u>			F
3. <u>S. faecalis</u> var. <u>casseliflavus</u>			
4. <u>S. faecalis</u> var. <u>liquefaciens</u>			F
5. <u>S. faecalis</u> var. <u>liquefaciens</u>			
6. <u>S. faecalis</u> var. <u>liquefaciens</u>			
7. <u>S. faecalis</u> var. <u>zymogens</u>			
8. <u>S. faecalis</u> var. <u>liquefaciens</u>			
9. <u>S. faecalis</u> var. <u>faecalis</u>			
10. <u>S. faecium</u>			
11. <u>S. faecalis</u> var. <u>zymogens</u>			
12. <u>S. durans</u>			
13. <u>S. faecalis</u> var. <u>liquefaciens</u>			
14. <u>S. faecalis</u> var. <u>zymogens</u>			
15. <u>S. bovis</u>			
16. <u>S. bovis</u>			
17. <u>S. faecium</u>			
18. <u>S. faecium</u>			G
19. <u>S. faecium</u>			G
20. <u>S. faecalis</u> var. <u>liquefaciens</u>			

NOTE:

FOR STRAINS 1-10 SEE FIGURE 3A, LANES 1-10; FOR STRAINS 11-20 SEE FIGURE 3C, LANES 1-10.

Plant Bioassays for the Detection of Environmental Mutagens

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ABSTRACT

It is well established that higher plants provide valuable assay systems for screening and monitoring environmental chemicals (de Serres 1978; Grant 1978, Grant et al. 1981; Nilan 1978). This project "Plant Bioassays for the Detection of Chemical Mutagens" was initiated in order to have a different relatively inexpensive mutagen assay system (using higher plants) which would provide a measurement of mutagenic toxicity in assay systems not being carried out by the Ontario Ministry of the Environment. This study involves the use of two higher plants, namely, Tradescantia and Barley (Hordeum vulgare). The Tradescantia assay systems are: (1) the Stamen Hair Assay for the detection of gene mutations, and (2) the Micronucleus Test for the detection chromosomal aberrations. The assay system to reported in barley is the Root Tip Chromosomal Aberration Assay. Ethyl methanesulfonate (EMS) is being used as a positive control. Tests are being completed on three herbicides, namely, alachlor (Lasso), atrazine, and metolachlor. Preliminary results indicate all three herbicides give a positive response in these plant assays. The herbicide atrazine is strongly mutagenic. The number of mutations from a dose of 50 ppm exceeded the number produced by a 250 ppm dose of EMS used as a positive control. Metolachlor also is mutagenic with the number of mutagenic events from a 240 ppm treatment exceeding the equivalent of 250 ppm EMS. In the stamen hair assay, the mutagenicity of Lasso was not significantly different from the control, however, Lasso caused a high percentage of chromosome aberrations in the micronucleus assay which was double those resulting from a 500 EMS treatment. Lasso also induced fragments in the barley chromosome aberration assay. From the chromosomal data, Lasso is considered to be a strong clastogen. The results to date indicate that higher plant assays are highly suitable for testing for the mutagenicity of environmental chemicals from air, soil and water habitats.

Introduction

Higher plants provide valuable assay systems for screening and monitoring environmental chemicals, both gaseous and liquid (Grant et

al. 1981). Although higher plant assays for the detection of mutagens have been in existence for many years, they have only recently received the recognition which these sensitive and reliable systems warrant (de Serres 1978; Grant 1978; Nilan 1978). As Stich and San (1980) stated "The recent successful introduction of the use of Tradescantia staminal hairs to detect airborne mutagens and carcinogens, may be the beginning of the recognition of various plant assays which are inexpensive, easy to handle and applicable to indoor as well as outdoor detection of environmental mutagens". Studies have shown that, for a specific chemical agent, comparable results in terms of genetic abnormalities are obtained in plant and animal systems. For example, in a survey of studies on the effects of pesticides, it has been shown that an excellent correlation exists between the frequency of both chromosomal abnormalities and C-mitoses in plant and animal systems (Grant 1978). A similar conclusion was drawn in studies on the effect of eight chemicals in several systems, including in vitro and in vivo mammalian systems, bacteria, Drosophila and plant systems. The plant systems showed excellent correlations with the mammalian systems (Clive and Spector 1978).

This project using higher plant assays was initiated in order to complement information provided by other mutagen assay systems being carried out by the Ontario Ministry of the Environment. In this study, two higher plant systems are being used:

- (1) Tradescantia clone 4430, heterozygous for flower color, blue dominant to pink recessive, developed at the Brookhaven National

Laboratory originally for the study of the effects of irradiation
(Underbrink et al. 1973)

Assays: (a) The staminal hair assay system will detect
air borne, soil, and aqueous mutants
(Ichikawa, S. 1978; Schairer et al. 1978)
(b) The micronucleus assay will detect
chromosome aberrations (Ma 1981).

(2) Hordeum vulgare (barley):

Assay: Root tips for the detection of mitotic
chromosome aberrations (Wuu and Grant 1966).

Protocols

Protocols have been fully developed for both the Tradescantia and Barley assays. A brief outline of these assays is given below and in greater detail in Tables I, II, and III.

Tradescantia

Tradescantia plants propagate readily, and initial emphasis was placed on the use this plant as a test organism as it is highly sensitive to chemical mutagens.

For the Stamen Hair Assay, inflorescences of the appropriate age are placed in a drop of paraffin oil and observed under a dissecting microscope. Pink mutant cells are scored. Details of the protocol are given in Table I.

For the Micronucleus Assay, pollen mother cells which are highly synchronized in *Tradescantia* are examined in the quartet (tetrad) stage (about 24 hours prior to anthesis) and micronuclei are scored. The micronuclei reflect chromosome aberrations which occurred at earlier stages in meiosis. The frequency of micronuclei per 100 cells can be used as an index of mutation. Details of the Protocol are given in Table II.

Barley

Barley seed is germinated in petri dishes in an incubator at 22 C. Seeds are treated and root tips of young seedlings are examined for chromosome aberrations. The protocol for the Barley Chromosome Aberration Assay is given in Table III.

Results to date

Tradescantia

Ethyl methanesulfonate (EMS)

A number of tests have been carried out using EMS as a positive control, since this chemical has been shown previously to give a positive response in both the *Tradescantia* Stamen Hair (ST) and Micronucleus (MCN) assays.

The results of several experiments are given as examples of our EMS studies which were carried out to validate the assay systems.

Stamen Hair Mutation Assay

In general, the mutation rate in stamen hairs with EMS is well above control values (Fig. 1). Tables IV is a representative experiment with 500, 1000, and 1500 ppm. Fig. 1 illustrates the results given in Table IV, and Fig. 2 the results of the EMS treatment minus the control. As may be seen (Fig. 2), there is only a slight increase in the mutation rate at the 1500 ppm concentration over the 1000 ppm. It may be that 1500 ppm is approaching the onset of toxicity.

Tradescantia Micronucleus (MCN) Assay and EMS Treatments

Tables V presents data on 25 and 100 ppm EMS treatments. As may be seen from this table, there is a strong positive response with EMS in the MCN test.

Results with Pesticides (Herbicides)

Atrazine

Representative results for treatments with the herbicide atrazine in the Tradescantia Stamen Hair assay are shown in Table VI and in Fig. 4. EMS at a concentration of 250 ppm is included as a positive control. The 50 ppm treatment gave almost double the number of mutations of the 250 EMS control (Table VI). The 25 and 50 ppm treatments gave a positive dose response, but the 100 ppm treatment, although above the control value, indicated that this concentration was approaching the toxic limit.

Alachlor (Lasso)

Representative results of treatment with Lasso in the stamen hair assay are given in Table VII and Fig. 5, along with a 250 ppm EMS treatment as a positive control. Results for the Micronucleus Test are given in Table VIII and in Fig. 6. As may be seen in Table VII, the results of all treatments were above both the water control and the EMS positive control. All treatments gave over double the number of mutations observed for the control. From the data presented, it may be seen that Alachlor induces both gene mutations and chromosome aberrations.

Metolachlor

Representative results for treatments with the herbicide metolachlor for the Micronucleus assay are given in Table IX. In Table IX, results of 240, 480 and 960 ppm treatments are given along with 500 ppm EMS as a positive control. As may be seen from this table and Fig. 7, only the 960 ppm treatment is strongly positive (over double the control). The 240 and 480 ppm treatments do not differ significantly from the control.

Barley

Some representative results of chromosome aberrations using barley as the test organism are given in Tables X and XI. As expected, a positive dose response was obtained for chromosome aberrations with EMS (Table

X). Chromosome aberrations included: stickiness, breaks, bridges, lagging chromosomes and polyploid cells.

Alachlor (Lasso): From the data (Table XI), it may be seen that Lasso causes chromosome aberrations and induces chromosome fragmentation (500 ppm for 3 and 6 hours treatments). The concentration of 1500 ppm for 24 hours was toxic and inhibited cell division.

Conclusion on the Mutagenic Effect of the Herbicides Tested

Atrazine

From the data presented it may be seen that Atrazine causes gene mutations. A 50 ppm treatment gave over three times the number of mutations as the control (Table VI). Atrazine has previously been shown to induce reverse mutations in the waxy locus of corn (Plewa and Wagner 1981). Atrazine has been reported to cause chromosome aberrations in plants (Wuu and Grant 1966, 1967).

The true mutagenic effect of atrazine may not be the result of treatment with atrazine per se, but a metabolic product which is produced in plants and which causes its mutagenic/chromosome aberration effects (Gentile et al. 1978; Plewa 1978).

Alachlor (Lasso)

Lasso caused both gene mutations (Table VII) and chromosome aberrations in *Tradescantia* (Tables VII, VIII) and in barley (Table XI). Lasso has been reported to cause reverse mutations in the waxy locus in

corn (Plewa and Wagner 1981) and chromosome aberrations in barley (Singh et al. 1978).

Metolachlor

Metolachlor was only slightly positive in the production of mutations in the stamen hair assay, but was quite positive for the production of chromosome aberrations in the micronucleus test (Table IX).

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TABLE I. Protocol for the Tradescantia Stamen Hair (ST) Assay

The Tradescantia Stamen Hair Assay was developed by A. H. Sparrow of Brookhaven National Laboratory for the study of the effects of ionizing radiation. Early radiobiological data demonstrated that stamen hairs were sensitive to as little as 0.25 rad of x-rays. Stich and San (1980) have stated "The recent introduction of the use of Tradescantia staminal hairs to detect airborne mutagens and carcinogens may be the beginning of the recognition of various plant assays which are inexpensive, easy to handle and applicable to indoor as well as outdoor detection of environmental mutagens."

For environmental chemicals, the Tradescantia clone 4430 has been developed at the Brookhaven National Laboratory. This clone is heterozygous for flower color and staminal hairs. Mutations are detected by the change in color from blue to pink.

The Staminal Hair Bioassay involves:

- a) Making cuttings of young inflorescences prior to meiosis.
Grow cuttings in aerated Hoagland's nutrient solution.
- b) Expose the young inflorescences by placing the cuttings in the test chemical and allowing the liquid to absorb through the stem to the inflorescence. One flower will develop about 400 stamen hairs.
- c) Collect flowers daily from 8 to 20 days after treatment (if chemical causes severe toxic effect flower development may be delayed, then examine as long as 25-30 days after treatment). Put flowers in a container with moistened sponge in refrigerator until ready to examine.
- d) Prepare slides by placing a stamen in liquid parafin for microscopic examination; add cover slip. Examine immediately if possible, or place in refrigerator for same day examination.
- e) Cytological Examination: Examined at a magnification of 400X (10 X ocular and a 40 X objective). Use white light source (no filters) for the detection of pink mutations.
- f) Statistics: Examine and score as in the following example:
Score 18 stamens per treatment per day from 18 different cuttings.
Score daily as above. Mutations are recorded as pink events per 100 or 300 stamens.

A complete description of the technique with illustrations are given in the paper by Schairer et al. (1978) and Underbrink et al. (1973). References are given at the end of the report.

TABLE II. Protocol for the *Tradescantia*
Micronucleus (MCN) Bioassay

The meiotic pollen mother cells (PMC) of *Tradescantia* are highly synchronized and are very sensitive to physical and chemical mutagens (Ma, 1981). A high frequency of acentric fragments can be induced with very low level of mutagens. These induced chromosome aberration become micronuclei (MCN) in the synchronized tetrads and they can be easily identified and scored.

The MCN Bioassay involves:

- a) Making cuttings of young inflorescences prior to meiosis. Grow cuttings in aerated Hoagland's nutrient solution.
 - b) Exposure of PMC in the young inflorescence of the plants by placing the cuttings in the test chemical and allowing the liquid to absorb through the stem to the inflorescence just prior to the onset of meiosis.
 - c) Fix flower buds 9 to 12 days after exposure (testing one or two buds prior to fixation to confirm that meiosis has proceeded to the tetrad stage) in acetic-alcohol (1:3).
 - d) Prepare microslides by removing a few PMc's at a time and squashing the cells in aceto-carmin stain. The stain penetrates fairly readily, but flaming over an alcohol lamp briefly will speed the staining process.
 - e) Cytological Examination: The tetrads are examined at a magnification of 400X (10 X ocular and a 40 X objective).
 - f) Statistics: An examination of 300 tetrads per slide and five slides per treatment have been found to give highly significant results.
-

A complete description of the technique with illustrations are given in the paper by Ma (1981). Earlier references are given in Ma's paper. Reference is given at the end of the report.

Table III. Protocol for the Barley Chromosome Aberration Assay

-
- 1a. Barley seeds, 50 seeds per petri plate are prepared and treated* without presoaking.
 - 1a. Tradescantia cuttings are made and placed in beakers. Beakers containing the treatment solutions are aerated with an air pump (aquarium pump). During treatment, the beakers are covered with aluminum foil and sealed with parafilm.
 2. Treatment
 3. Washing
 4. Recovery, 24 hours at 20 C.
 5. Harvest roots
 6. Pretreatment: 0.003 M 8-hydroxyquinoline, 1-3 hours
 7. Fixation, Carnoy's (3:1, 95% ethanol-glacial acetic acid), at 0-4 C for 12-24 hours (16 h)
 8. Hydrolysis, 1N HCl at 60 C for 10 min for root tips and 20 min for flower buds
 9. Stain, basic fuchsin for 1 hour at 30 C in the dark.
 10. Destaining, 3 changes of 50% water for 15 min each.
 11. Storage, 70% ethanol in a refrigerator
 12. Preparation: Rinse in water, 3 changes
 13. Maceration: 4% pectinase, 1 to 3 hours
 14. Slide preparation: Squash in 45% acetic acid
 - 15a. Temporary slide: seal with rubber cement
 - 15b. Permanent slide: Slide is placed on dry ice; remove cover slip with razor blade; root tip material adheres to slide; immerse in 2 changes of absolute alcohol and mount in Euparal, Permount or equivalent.
-

*For those chemicals which are not water soluble, the chemical is dissolved in dimethyl sulfoxide (DMSO). Treatments are applied for a period of 6 to 24 hours with a 24 hour recovery period.

Table IV. *Tradescantia* Stamen Hair Mutation Assay, 6 hour treatment with ethyl methanesulfonate (EMS)
Prepared 291085

Dosage (ppm)	No. flowers scored	No. of hairs	Number of Pink events	Mutations	Scoring
				No. /100 hairs	period
0	43	16,770	96	0.57 + 0.29	141085-291185
500	46	16,560	183	1.10 + 0.46	111085-281185
1000	30	10,620	148	1.39 + 0.78	121185-281185
1500	32	12,288	175	1.42 + 0.89	111185-271185

Table V. *Tradescantia* Micronucleus (MCN) Mutation Assay, 6 hour treatment with ethyl methanesulfonate (EMS), 2.5 days recovery.
Prepared: 050386.

Dosage (ppm)	Number of MCN/300 Tetrads	Number of Tetrads scored	Mean number of MCN/300 Tetrads	Number of cells with MCN/300 tetrads	Mean no. cells with MCN/300 tetrads
0	20	2,297	2.5 + 1.7	15	1.88 + 1.2
25	121	2,400	15.1 + 5.6	116	14.50 + 5.1
100	270	2,993	27.0 + 9.6	234	23.40 + 3.2

Table VI. *Tradescantia* Stamen Hair Mutation Assay, 6 hour treatment with ATRAZINE and 250 ppm ethyl methanesulfonate (EMS) as positive control. Prepared: 060586

Dosage (ppm)	No. flowers scored	No. of hairs	Number of Pink events	Mutations	Scoring period
				No. /100 hairs	
0	28	8,568	70	0.82 + 0.10	120586-290586
250 EMS	34	9,588	192	2.00 + 0.93	140586-290586
25	43	12,384	171	1.38 + 0.57	190586-290586
50	44	13,728	483	3.52 + 1.48	150586-290586
100	22	6,072	75	1.24 + 1.12	120586-290586

Table VII. *Tradescantia* Stamen Hair Mutation Assay, 6 hour treatment with ALACHLOR (LASSO); 250 ppm ethyl methanesulfonate (EMS) used as positive control. Prepared: 120586.

Dosage (ppm)	No. flowers scored	No. of hairs	Number of Pink events	Mutations	Scoring period
				No. /100 hairs	
0	16	4,992	49	0.98 + 0.47	210586-050686
250 EMS	22	5,808	115	1.98 + 1.12	220586-050686
300	37	11,100	230	2.07 + 0.77	220586-050686
500	33	9,504	259	2.72 + 1.37	210586-050686
700	24	8,064	202	2.50 + 1.32	260586-050686

Table VIII. *Tradescantia* Micronucleus (MCN) Mutation Assay, 6 hour treatment with ALACHLOR (LASSO); 500 ppm ethyl methanesulfonate (EMS) used as positive control. Prepared: 041085.

Dosage (ppm)	Number of MCN/300 Tetrads	Number of Tetrads scored	Mean number of MCN/300 Tetrads
0	41	4,565	2.73 + 0.64
500 EMS	53	4,799	3.31 + 0.55
125	102	4,175	7.28 + 2.44
250	70	3,570	5.83 + 1.81
500	51	3,769	4.25 + 1.00
1000	78	3,900	6.00 + 1.86

Table IX. *Tradescantia* Micronucleus (MCN) Mutation Assay, 6 hour treatment with METOLACHLOR; 500 ppm ethyl methanesulfonate (EMS) used as positive control. Prepared: 020586.

Dosage (ppm)	Number of MCN/300 Tetrads	Number of Tetrads scored	Mean number of MCN/300 Tetrads
0	1.0	3,600	0
500 EMS	26	3,781	2.00 + 0.57
240	21	3,300	1.91 + 0.81
480	18	4,200	1.29 + 0.83
960	45	2,941	4.50 + 2.68

Table X. Barley chromosome aberration assay. Treatment with the positive mutagen, ethyl methanesulfonate (EMS)

Dosage (ppm)	Time (h)	No. dividing cells	Total no. cells	Mitotic index* $\times 10^{-3}$	Total aberrations	Types
0	3	18	3555	5.06	0	
500	3	15	4835	3.1	3	Lagging chromosomes, clumping, spindles
500	3	40	1648	2.43	19	Breaks, laggards, fragments; polyploidy
1000	3	10	3789	0.264	1	Clumping
1000	3	6	3084	1.95	5	Stickiness, laggards fragments, micronuclei
1500	3	39	3258	1.20	4	Bridges, laggards, clumping
0	6	24	3400	7.59	0	
500	6	29	3804	7.62	3	Clumping
500	6	15	2784	5.39	5	Bridges, stickiness, laggards
1000	6	10	5440	1.84	4	Stickiness, polyploidy
0	24	188	6630	28.4	0	
500	24	21	2632	7.98	3	Chromosome breaks, polyploidy
1000	24	23	6720	3.42	4	Stickiness, clumping
1000	24	54	3584	1.51	7	Fragments, spindle, polyploidy
1500	24	44	1792	2.46	2	Telophase bridges

*Mitotic Index: indicates the fraction of the total number of cells scored in any stage of mitosis.

Table XI. Barley chromosome aberration assay; treatment with
ALACHLOR (Lasso)

Dosage (ppm)	Time (h)	No. dividing cells	Total no. cells	Mitotic index $\times 10^{-3}$	Total aberra- tions	Types
0	3	121	15708	7.70	1	Fragment
0	6	19	2634	7.21	0	
0	24	17	2870	5.92	0	
500	3	10	1760	5.68	4	Fragments
500	6	11	4149	2.65	2	Fragments
1000	3	20	1634	1.22	4	Fragments; stickiness
1500	3	16	1888	8.47	2	Fragments
1500	6	8	1649	4.85	0	Toxic, cells killed
1500	24	0	5196	0.00	0	Toxic, cells killed

Fig. 1.

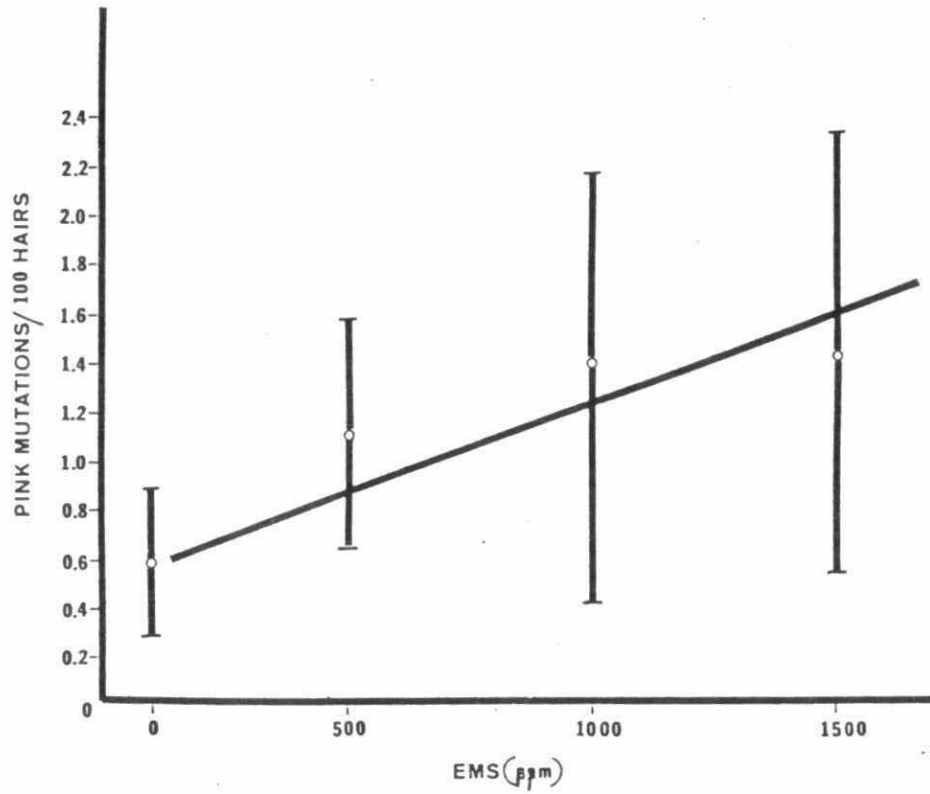
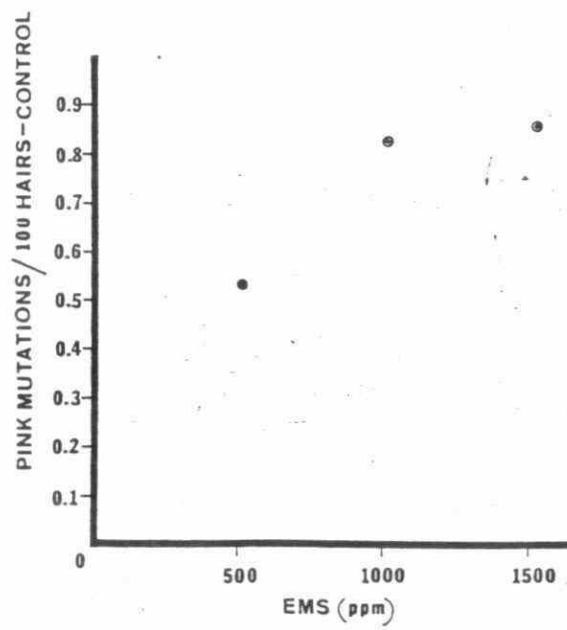


Fig. 2.



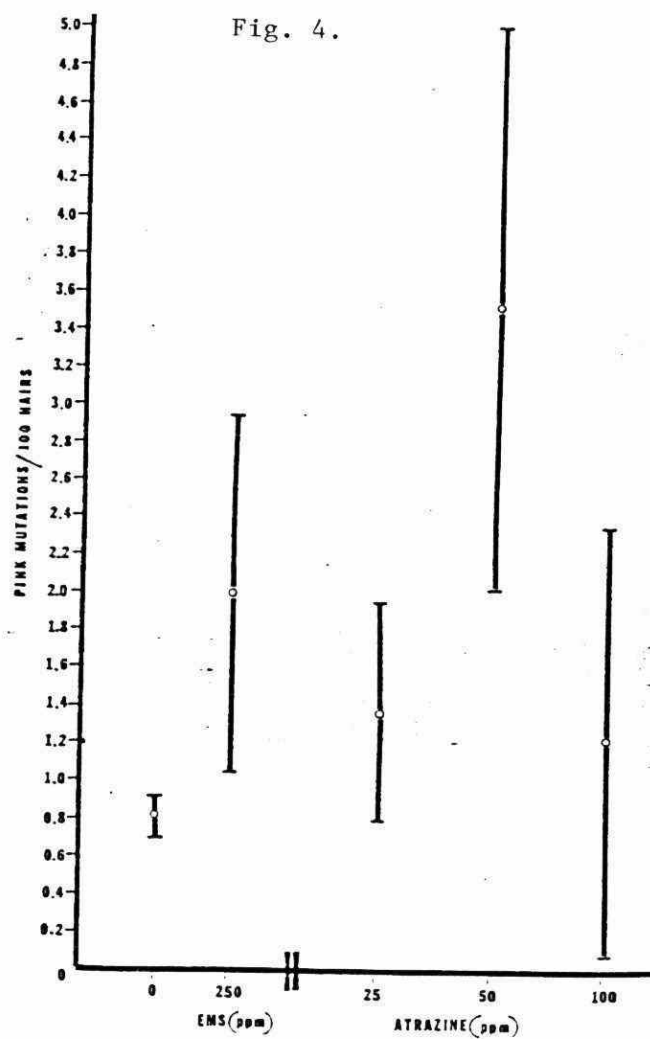
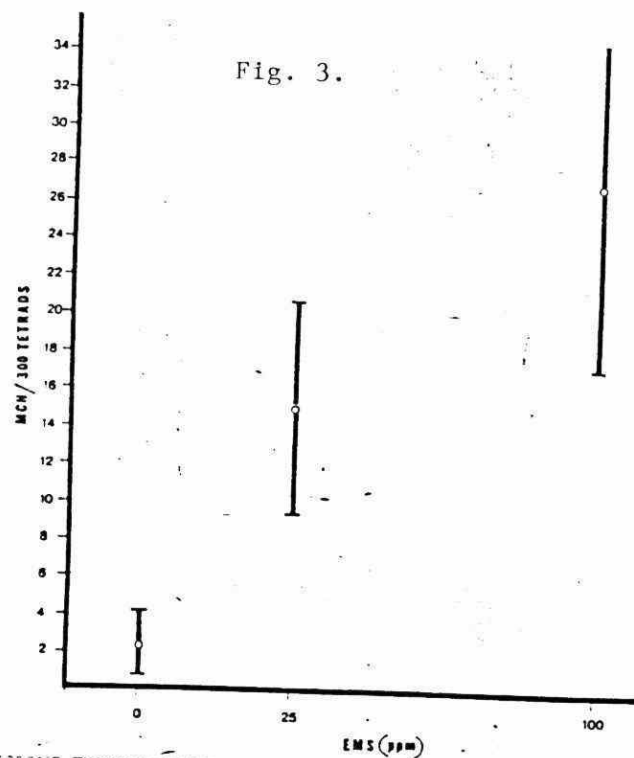


Fig. 6.

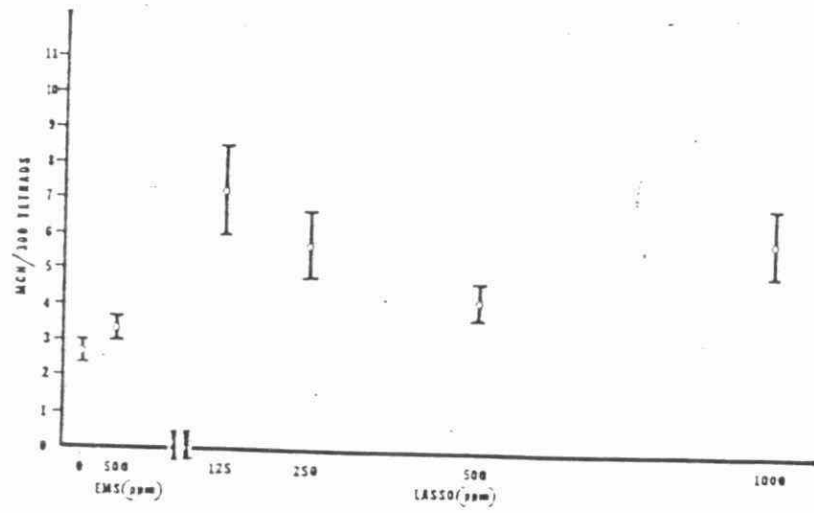


Fig. 7.

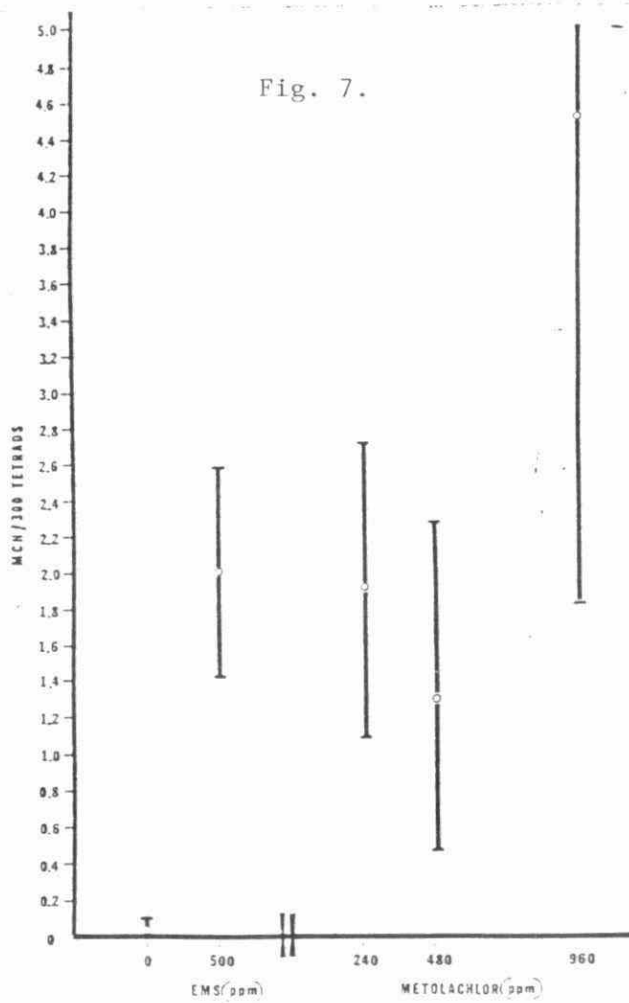
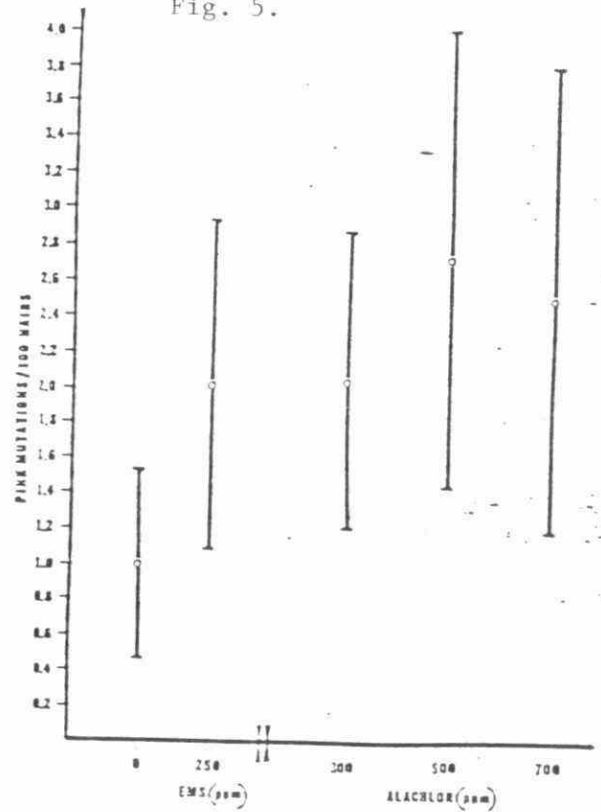


Fig. 5.



A BIOLOGICAL INDICATOR SYSTEM TO IDENTIFY GENOTOXICITY OF IN-PLACE POLLUTANTS

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INTRODUCTION AND BACKGROUND

In recent years, the occurrence of hyperplasia and neoplasia in fishes from the Great Lakes and other waters has received widespread attention as a possible indicator of water quality problems (see reviews by Baumann 1984 and Black 1984). There is circumstantial evidence that morphological abnormalities may also develop in benthos in response to sediment contamination. Most studies on abnormalities in freshwater macroinvertebrates have examined the occurrence of deformities in Chironomidae.

Brinkhurst et al. (1968) and Hamilton and Saether (1971) reported that specimens of Chironomus spp. collected from the western end of Lake Erie near the Maumee River mouth were badly deformed. The most conspicuous feature of these larvae was an exceedingly thick exoskeleton. The head capsules were also thickened and heavily pigmented, and the mouth parts exhibited a variety of aberrations. Stations from the remainder of the lake yielded more than 1,700 specimens, all of which appeared normal.

Warwick (1980a) recovered remains of deformed chironomid larvae, with deformities similar to those described by Brinkhurst et al. (1968), from sediment cores collected in the Bay of Quinte, Lake Ontario. The percentage of deformed specimens of Chironomus and Procladius clearly increased in the most recent sediments. According to the sediment core chronology, the incidence of deformities increased from 0.09% in the pre-European sediments to 1.06% at 4.5 cm (1951) and 1.99% in the 1972 chironomid population.

In a study of the benthic community in Parry Sound, Georgian Bay, Hare and Carter (1976) found that 77% of Chironomus (s.s.)? cucini larvae had deformities of the mouthparts. Refuse entering Parry Sound Harbour during the past 100 years from lumbering, smelting and explosives industries, as well as urban sewage, has left the sediments visibly contaminated with oil, cinders and saw-mill leavings. The harbour was the site of a massive oil spill in 1950. Of 73 larvae examined from outside the harbour only one specimen was found to be slightly deformed.

Cook and Veal (1968) reported that most of the chironomids (Chironomus spp.) at a sampling station in the turning basin of Port Hope Harbour, near the cooling water

discharge of a uranium refining plant, had deformed labial plates similar to those described by Brinkhurst et al. (1968). McKee et al. (1985) and Warwick et al. (1986) reported a deformity frequency of 83% for Chironomus specimens collected in the turning basin compared with only 14% in the outer harbour. Sediment quality data showed that the inner harbour is heavily contaminated by U-238 decay chain radionuclides and several trace metals, particularly in the turning basin, while the outer harbour is relatively uncontaminated.

Several other investigators also have reported deformities in Great Lakes chironomids. Boscor et al. (1974) reported increased frequencies of deformed Chironomus in Black River Bay, Lake Ontario, and implicated pollution by agricultural and industrial toxicants as a probable causal factor. Crowther and Luoma (1984) reported the occurrence of deformed larvae of C. salinarius in a habitat impaired by pulp and paper mill discharges in Thunder Bay, Lake Superior. In a survey of benthic macroinvertebrates of the central basin of Lake Erie, Krieger (1984) reported that one specimen of Chironomus near the mouth of the Grand River at Fairport Harbor and two specimens of Procladius near the mouths of the Black River at Lorain and the Cuyahoga River at Cleveland possessed deformed menta or ligulae, respectively. The occurrence of deformed chironomids in polluted waters outside of the Great Lakes basin has been reported by Nelson and Blaylock (1963), Blaylock (1965), Hamilton and Saether (1971), Koehn and Frank (1980), Warwick (1980b), Tennessen and Gottfried (1983), Cushman (1984) and Wiederholm (1984).

These studies on the occurrence of deformed chironomids in areas affected by industrial and/or agricultural pollution suggest the hypothesis that sediment contamination may induce chironomid deformities, and the possibility that deformity frequency may be used as a sediment quality indicator. However, a number of questions remain to be answered. In particular:

1. Can deformities similar to those seen in the field be induced by life-cycle exposure of normal stock to known genetic toxicants?
2. To what extent do observed deformities have a heritable genetic basis?
3. Can deformities be induced by physical (e.g., grain size, temperature) as opposed to chemical factors?

These questions are fundamental to our understanding of a chironomid indicator system and what it may tell us about sediment quality, and are best addressed in controlled laboratory experiments.

The only previous laboratory experiments in which any of these questions have been addressed are those of Hamilton and Saether (1971). These authors exposed cultures of Chironomus tentans through one life cycle (egg to 4th instar) to lethal concentrations of aldrin, DDE, PCB's and 1:1 2,4-D:2,4,5-T. A small proportion ($\approx 10\%$) of the larvae surviving exposure to 10 ug/L of DDE in solution were found to be deformed with thickened body walls. Deformed mouthparts were not observed.

Cushman (1984) indicated that laboratory studies were necessary to establish causal relationships between deformities and particular toxicants, e.g., with larvae from single egg clusters reared at different dose levels, so that the exposure histories of the larvae and their parents are known. The laboratory studies described in this report were designed to determine whether mouthpart deformities could be induced in chironomids by a known genotoxicant, ethyl methane sulfonate (EMS), either in exposed organisms or in their unexposed offspring.

METHODS

Culture Conditions

A stock culture of Chironomus tentans was established from ten egg masses collected in the field by B.E. Townsend, Freshwater Institute, Department of Fisheries and Oceans, Winnipeg, Manitoba. The stock culture was maintained in the laboratory for three generations prior to initiating experiments on deformity induction. Culture methods follow Townsend et al. (1981) and Batac-Catalan and White (1982). Air temperature was maintained at $25^{\circ} \pm 2^{\circ}\text{C}$ and photoperiod at 16L:8D.

The culture was reared in a 40-L glass aquarium with 5 cm of fine sand on the bottom. The aquarium was supplied with carbon-filtered tap water at a rate of 0.5l/hr and a water level of 10 cm was maintained by a standpipe drainage system. The water volume was 15L. Aeration was provided by a single airstone. The culture was fed ad libitum with a slurry of either Cerophyl, a dried plant product, or Tetramin fish food, every three days. The amount increased with the stage of development and food requirements of the culture, and was sufficient to prevent the formation of food-clear areas around the developing larvae. Excess food and debris were gently siphoned from the bottom before each feeding.

The aquarium was covered with a fine mesh screen in order to prevent the escape of emerging adults. Adults were collected from the aquarium by aspiration into an Erlenmyer flask containing fresh water to a depth of 3 cm. They were confined to the flask by a cotton wool stopper, in a sex ratio of 2 males to 10 females, and left overnight

to mate. Egg masses collected the following morning were used to start a new stock culture with clean sand and water.

Experiments were conducted under conditions similar to those used for stock culture maintenance, except that each egg mass was reared in a separate I-L culture bowl with its own air supply. Water or test solution was siphoned out of the bowl, almost to substrate level, and replaced prior to feeding every three days. Tetramin was used as a food source during preliminary toxicity experiments, while Cerophyl was used in subsequent experiments on deformity induction. Tetramin was prone to fungal growth under semi-static culture conditions.

EMS Toxicity Determinations

Preliminary toxicity experiments were conducted in order to estimate the LC50 concentrations of EMS and establish a sublethal dose range for future experiments on deformity induction. Replicate test cultures were established at EMS concentrations of 0, 50, 100, 500, 1,000 and 5,000 mg/L. Exposure was initiated on the day following egg mass deposition and continued through the 4th instar stage. The 4th instar larva is 12-24 mm in length, bright red in colour, and distinguished from previous instars by a dark dorsal head stripe.

The number of eggs in each egg mass was counted under a dissecting microscope prior to placement of the egg mass in the test solution. The culture was decanted and preserved in 10% formalin when the first pupae were observed, and then washed through a 500 um mesh screen. Larvae and pupae were counted, and divided by the initial egg count to give a survival estimate.

The approximate LC50 concentration of EMS was estimated as the midpoint between two adjacent exposure levels, marked by apparently normal survival at the lower level (100 mg/L) and complete mortality at the next higher level (500 mg/L). Confidence limits could not be determined due to the lack of a graded response series at intermediate exposure levels.

Determination of Sublethal Effects

Experiments on deformity induction were conducted at exposure levels of 0, 50, 75, 100 and 240 mg/L, with two replicate cultures at each concentration. Exposures spanned the period from egg mass deposition through 4th instar, as previously described. However, some larvae from each culture were allowed to complete pupation and emerge as adults. Culture bowls were covered with a fine screen mesh to prevent adults from escaping. Emerging males were collected each day for several days and mated with

virgin females from the control cultures, in order to determine from their offspring whether heritable genetic damage had been induced. Then both exposed and control cultures were preserved as previously described.

Virgin females were isolated by placing individual pupae just prior to emergence in individual glass jars containing clean water and covered with a fine mesh screen. Some of the adults emerging in these jars were males and were discarded. Others were females and were paired with individual males from exposed cultures in Erlenmeyer mating flasks. Each egg mass deposited was counted and placed in a separate I-L culture bowl, labelled according to the exposure history of the parents. These cultures were reared through 4th instar without exposure to EMS, and then preserved.

Preserved organisms from each culture, whether they had been directly exposed, derived from exposed male parents, or controls, were washed, sorted and counted as previously described. Counts of larvae, pupae and adults were divided by initial egg counts to estimate culture survival. Fourth instar larvae were blotted on tissue paper and weighed en masse to determine average weight for each culture. One hundred 4th instar larvae from each culture, or the total number if less than 100, were cleared and mounted for microscopic examination of their mouthparts.

Clearing and mounting techniques followed Warwick and Casey (1982), except that only the head capsules were mounted. Head capsules were severed from the body after clearing in cedar oil and prior to mounting in Canada Balsam. The bodies from each culture were stored in 70% ethyl alcohol.

Scoring of mouthpart deformities was conducted by double blind microscopic examination of the hypostomial plate, after each investigator had been 'trained' on the same series of sample slides designated normal or abnormal. Normal mouthpart anatomy is illustrated in Figure 1. Abnormalities included the following types:

1. missing or fused lateral teeth;
2. extra lateral teeth;
3. wide gaps between two teeth (the 'Koehn' gap);
4. missing or fused processes of the median tooth (normally trifid);
5. extra processes or dissection of the median tooth;
6. missing or fused processes of the first lateral tooth (normally bifid); and
7. extra processes of the first lateral tooth.

Typically, abnormalities were assymetrical, except in several control cultures. In one, a symetrical partial fusion of processes on both the right and left first lateral

tooth was common while, in the other, a cleft median tooth was common. These symmetrical characteristics were scored as abnormalities. Cases of obvious physical damage to the teeth, while rarely observed, were excluded from the abnormality count.

The abnormality frequencies determined by two investigators were averaged to compute a final abnormality score for each culture examined. Investigators agreed on 90% or more of the slides examined from each culture, except for a concordance of only 78% on the control with symmetrical partial fusion of processes on the first lateral.

RESULTS AND DISCUSSION

Chronic Embryo-Larval Toxicity

Preliminary experiments on embryo-larval toxicity of EMS were undertaken to establish a sublethal exposure range for later experiments on deformity induction. Preliminary exposures spanned two orders of magnitude from 50 mg/L to 5g/L. Survival from egg to 4th instar is shown in Table 1 for exposed and control cultures.

Low survival in control cultures (20 to 26%) may be related to loss of temperature control at one point prior to hatching when temperature fell overnight from 20°C to about 3°C. Townsend *et al.* (1981) report hatching failure at a temperature of 8°C throughout embryonic development, and Sadler (1935) reports developmental arrest in 4th instar at constant temperatures less than 8°C. Apparently normal hatching occurred in control cultures and those exposed to EMS at low levels.

Cultures at 100 mg/L and higher exposure levels were not followed beyond 4th instar in this experiment. However, adults were allowed to emerge and mate at 50 mg/L as a check on reproductive success.

At 100 mg/L EMS survival to 4th instar was apparently normal. At 500 mg/L EMS complete mortality occurred following hatching. Hatching was confirmed after disintegration of the egg mass, by microscopic examination of substrate samples, which contained large numbers of first instar larvae. No attempt was made to count these larvae and none survived to the visible 2nd instar stage.

At 1 g/L and higher levels of EMS hatching did not occur. Eggs turned white within the egg mass, which then failed to disintegrate normally. The culture was disposed of after 5 days, at which time no eggs with normal colouration were present.

On the basis of these experiments the LC50 for embryo-larval survival through 4th instar was estimated at approximately 300 mg/L EMS, although in the absence of intermediate exposure levels producing a partial mortality response, the actual LC50 may be anywhere in the range from 100 mg/L to 500 mg/L.

Survival through Emergence

A second series of embryo-larval exposures were conducted in the range from 75 mg/L to 400 mg/L EMS, in order to observe sublethal effects. Numbers of chironomids at each stage of development when cultures were preserved are shown in Table 2. One replicate at each exposure level was preserved when adults first began emerging; the other approximately one week later. Adults which emerged successfully are included in the table, even though they were not preserved with the culture. Adults which died while emerging or within hours of emerging are not included.

Control survival was better in these experiments (49-80%) than in the preliminary toxicity experiments, in spite of the later developmental end points used. Improved survival was probably due to the more stable thermal regime. The use of Cerophyl as opposed to Tetramin for feeding may also improve survival in semi-static culture.

Death at emergence was a common syndrome at 100 mg/L where most of the adults which began to emerge failed to complete the process. They seemed unable to detach themselves from the pupal case, an exercise which requires vigorous muscular action. Emerging adults were much less active in general at this exposure level than in control cultures. Dead midges attached to their pupal cases were also common at 75 mg/L.

Post-emergence mortality was the predominant adult mortality syndrome at 75 mg/L. Many adult midges would shed their pupal case, but drown before the daily collection from the culture. This also occurred in control cultures, but not with the same frequency since the majority of control adults were collected alive.

At 240 mg/L no adults emerged although many larvae pupated in apparently normal fashion.

At 400 mg/L only 10 larvae reached the 4th instar stage, out of 4200 eggs in two cultures. None of these larvae either formed tubes in the substrate or pupated. Numbers of survivors at this dose level were considered insufficient for determination of larval weight or deformity incidence.

Weight of 4th Instar Larvae

Average larval weight was determined in cultures exposed to EMS at concentrations between 50 mg/L and 240 mg/L. Larval weight was greatest in cultures exposed at 100 mg/L EMS, and lowest in the control cultures. Table 3 illustrates an increase in larval weight up to the 100 mg/L dose level, followed by a weight decline at

240 mg/L. The weight increase with dose level is probably the result of decreasing culture densities (Table 2) associated with reduced survival. The decline in weight at 240 mg/L may reflect toxic effects of EMS, or simply an additional manifestation of the density effect. Larval density was greater at 240 mg/L than at 100 mg/L, at least in part due to the greater numbers of eggs in the initial egg masses at 240 mg/L.

The normal average weight for late 4th instar larvae, according to Townsend et al. (1981), is 22 mg for male larvae and 37.7 mg for female larvae. Both male and female larvae contribute to the weights shown in Table 3. While the sex ratio was not determined, average weights between the normal male and female averages would be expected in the absence of crowding. This weight range was achieved only in 100 mg/L cultures, suggesting possible crowding effects in the other cultures. Crowding probably contributes to the wide range of developmental stages observed at the time of culture preservation.

Larval Deformities

The frequency of 4th instar larvae with abnormalities of the hypostomial plate was determined in cultures exposed to EMS at concentrations between 50 mg/L and 240 mg/L, and in control cultures. The 50 mg/L culture was established as part of the chronic toxicity experiment. Thus two sets of control cultures were scored for hypostomial deformities, one set concurrent with the 50 mg/L exposure, the other concurrent with exposures from 75 mg/L to 240 mg/L.

Deformity incidence is summarized in Table 4 for the exposed generation, and for the offspring of parents exposed at 50 mg/L. Both parents of this F_1 culture had been exposed to EMS. Thus it is not possible to rule out cytological effects of EMS on the developing ova of female parents, or to distinguish such cytological effects from genetic effects in the F_1 culture. The deformity incidence in this F_1 culture (33%) was greater than in any control culture, and greater than in the parental cultures at 50 mg/L, suggesting an EMS effect due to parental exposure. However, due to a fairly high and variable control incidence, and lack of replication of F_1 cultures, this result is not considered conclusive.

The effects of EMS on morphology of exposed larvae were more demonstrable. Deformity incidence was dose related and doubled relative to controls at 100 mg/L and 240 mg/L (Figure 2). The deformity incidence differed significantly from concurrent controls at 240 mg/L, based either on a student's t-test comparing mean deformity frequencies after angular transformation, or on chi-square tests comparing individual replicates ($p < 0.05$ in both cases).

The types of deformities observed are categorized in Table 5 for each culture. These data suggest a tendency to certain types of abnormalities in different cultures. This tendency was particularly evident among control cultures and may be related to the individual genetics of the parents producing each egg mass.

For example a predisposition toward cleft median teeth was seen in Control 1, where this specific condition accounted for 10 of the 19 hypostomial abnormalities observed. A predisposition toward partial fusion of the two processes of the first lateral tooth was seen in Control 2. The fusion was seldom complete, and was variable in degree. Arbitrarily, teeth showing an acute angle between the two processes were considered normal; those showing less separation were considered abnormal.

The conditions described above in Controls 1 and 2 were highly symmetrical. The cleft in the median tooth usually divided it into two equal lobes, and similar degrees of fusion were observed in right and left first lateral teeth. While clearly abnormal, the symmetry of these conditions was consistent with an inherited genetic basis. Somatic damage is much less likely to be symmetrical.

Hare and Carter (1976) considered only asymmetrical defects of the mentum in estimating deformity incidence in chironomids from Perry Sound Harbour. We have included symmetrical defects in our estimates of deformity frequency. Symmetrical defects were rare in EMS-exposed cultures. However, their exclusion would approximately halve the deformity frequency reported for Controls 1 and 2 in Table 4.

The frequency of deformities observed in control cultures was higher than that typically reported for field collections of chironomids from pristine locations. Frequencies in such field collections have seldom exceeded 1%. As discussed above, high control frequencies may be related to parental genetics. Inbreeding of the stock culture may be expected to result in increased expression of recessive characteristics, including morphological abnormalities with a heritable genetic basis. Evidence that this may be a relevant factor was provided by deformity incidence monitoring of the stock culture prior to the experiments with EMS.

A trend to increasing deformity incidence between the fourth and sixth generations in captivity was suggested (Figure 3). The experiments with EMS were conducted with fifth and sixth generation stock. Batac-Catalan and White (1982) recommend replacement of stock cultures every seven generations in order to ensure culture productivity, but do not discuss morphological condition.

Other explanations for high control deformity levels may include temperature, physical or crowding effects. Temperature control was lost at one point in the chronic toxicity experiments, which may have influenced deformity frequencies reported for

controls A and B and 50 mg/L exposures. Temperature fell overnight from 20°C to about 3°C. Townsend et al. (1981) report hatching failure at a temperature of 8°C throughout embryonic development, and Sadler (1935) reports developmental arrest in 4th instar at constant temperatures less than 8°C.

Physical effects may be associated with the substrate material or food used for laboratory culture. Both are probably coarser and harder than natural substrates and foods, and may be abrasive to chironomid mouthparts.

Crowding within the culture may also influence development, through general developmental rate effects, although there is no evidence to suggest a specific influence on mouthpart morphology. Initial egg densities in the experimental cultures were 5 to 10 times those recommended by Townsend et al. (1981), although Townsend does not specifically mention density effects on survival.

Finally, the higher incidence of deformities in experimental controls than in unpolluted field locations may be related to stronger natural selection against mouthpart deformities in field situations. Natural selective pressures related to mouthpart morphology of chironomids are virtually unknown.

Reproductive Effects in Adults

Males from exposed cultures at 75 mg/L and 100 mg/L were mated with virgin females from control cultures in order to determine reproductive success. Exposed males and exposed females were mated at 50 mg/L. The numbers of matings attempted and the number of egg masses obtained at each dose level are summarized in Table 6. Only the egg masses produced at 50 mg/L were viable.

Virgin females do not lay eggs (Townsend et al., 1981). Thus, the females producing eggs at 75 mg/L and 100 mg/L were inseminated by EMS exposed males, the only males with which they had come in contact. The lack of embryonic development could be due either to male infertility, or to expression of dominant lethal mutations from the male genome. We cannot distinguish between these alternatives since the early embryo was not examined microscopically for evidence of cell formation. Gene expression in an insect embryo begins in the syncytial blastoderm stage after nuclear migration but prior to cell formation (Marx, 1984). In Chironomus, this period lasts for about four hours after nuclear migration (Yajima, 1964).

Males exposed to EMS at 50 mg/L were fertile, as were females, although the level of mouthpart deformities in their offspring suggests that some genetic damage to the germ cells may have occurred. This mating was not designed to distinguish between male and female parental effects.

CONCLUSIONS

It has been demonstrated that hypostomial plate deformities similar to those seen in chironomids from polluted environments can be induced by a genotoxicant in the laboratory. This suggests a genetic basis for observed deformities. However, a number of questions pertinent to the interpretation of chironomid morphology as a sediment quality indicator remain to be answered. In particular:

1. To what extent are induced deformities heritable? While deformities induced at higher dose levels of EMS were not heritable due to poor emergence and reproductive inhibition, heritable effects may be induced at lower dose levels. These effects require further study since heritability and persistence of deformities in natural populations influence the temporal resolution of the system for in situ sediment quality monitoring.
2. Can physical damage in early instars produce deformities in 4th instar larvae which resemble those induced chemically? While obvious physical damage was rarely observed in 4th instar larvae, molting may obscure the physical nature of early instar damage, without eliminating the defect. Thus, studies of factors producing physical damage and of damage transmission between instars are warranted.

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TABLE 1: CHRONIC EMBRYO-LARVAL TOXICITY OF EMS

	Nominal ¹ Concentration of EMS (mg/L)											
	0		50		100		500		1,000		5,000	
	A	B	A	B	A	B	A	B	A	B	A	B
Approximate ² Initial No. of Eggs	1,725	1,750	1,425	2,075	2,025	2,275	2,300	2,050	1,900	2,275	2,325	1,700
Hatching Observed	+	+	+	+	+	+	+	+	-	-	-	-
2nd Instar Observed	+	+	+	+	+	+	-	-	-	-	-	-
Approximate Survival to 4th Instar ³ (%)	20	26	19	12*	23	15	0	0	0	0	0	0

¹ EMS degrades in water with a four-day half-life at 20°C; solutions were replaced every three days.

² Based on a count of 1/10th of the length of each egg string.

³ Based on counts of 3rd and 4th instar larvae at an endpoint marked by the first appearance of pupae.

* Emergence and mating was allowed in 50 mg/L culture B; thus, it was not preserved concurrently with the other cultures.

TABLE 2: LARVAE SURVIVING AND ADULTS EMERGING FROM CULTURES EXAMINED FOR DEFORMITY INDUCTION

EMS Concentration (mg/L)	Approximate Initial No. of Eggs	No. of Organisms at Preservation					% Survival
		2nd Instar Larvae	3rd Instar Larvae	4th Instar Larvae	Pupae	Adults*	
Replicate No. 1 (preserved 7 days in advance of replicate No.2)							
0	1,175	-	791	100	5	40	80
75	1,275	8	608	163	2	5	62
100	1,475	3	17	39	26	2	6
240	1,725	-	-	108	47	-	9
Replicate No. 2							
0	1,775	-	-	70	1	800	49
75	1,500	-	71	116	33	2	15
100	1,425	-	5	19	5	3	2
240	2,125	-	-	28	2	-	1

* Including adults removed alive prior to culture preservation.

TABLE 3: MEAN WEIGHT OF 4TH INSTAR LARVAE EXAMINED FOR DEFORMITY INDUCTION

EMS Concentration (mg/L)	Replicate Number	Approximate Initial No. of Eggs	No. of 4th Instar Larvae	Mean Larval Weight (mg)
0	1*	1,175	100	6.6
	2	1,775	70	11.1
75	1*	1,275	163	13.1
	2	1,500	116	13.4
100	1*	1,475	39	23.8
	2	1,425	19	20.5
240	1*	1,725	108	13.9
	2	2,125	28	**

* Replicate 1 was preserved 7 days in advance of replicate 2.

** Larvae not weighed prior to mounting.

TABLE 4: INCIDENCE OF MOUTHPART DEFORMITIES IN RELATION TO EMS EXPOSURE LEVEL

EMS Concentration (mg/L)	Replicate Number ¹	No. of 4th Instar Larvae Examined	No. of Larvae Deformed	% Deformed
0	A	100	14	14
	B	100	6	6
0	1*	95	19	20
	2	67	14	21
50	A	96	13	14
	B	99	13	13
75	1*	100	13	13
	2	99	30	30
100	1*	39	14	36
	2	19	8	42
240	1*	92	42	46
	2	28	14	50

¹ Cultures labelled A and B were initiated in preliminary toxicity determinations; those labelled 1 and 2 were initiated 30 days later.

* Replicate 1 was preserved 7 days in advance of replicate 2.

TABLE 5: TYPES OF DEFORMITIES OBSERVED IN 4TH INSTAR LARVAE EXPOSED TO EMS

Type of Deformity	Percentage ¹ of Deformed Larvae ² in Each Category											
	Control		Control		50 mg/L		75 mg/L		100 mg/L		240 mg/L	
	A	B	1	2	A	B	1	2	1	2	1	2
	(14)	(6)	(19)	(14)	(13)	(13)	(13)	(30)	(14)	(8)	(42)	(14) ²
1. Missing or fused lateral teeth	43	-	5	29	15	23	69	53	43	63	26	7
2. Extra lateral teeth	7	-	5	-	-	-	15	-	7	-	-	-
3. Koehn gap	-	-	-	-	8	-	-	-	7	-	-	7
4. Abnormal ³ median tooth	50	83	84*	14	62	38	15	40	21	75	60	71
5. Abnormal ³ first lateral tooth	-	33	16	57**	38	62	8	13	36	-	14	29

¹ Column sums of percentages may exceed 100% since some larvae displayed multiple types of abnormalities.

² Numbers in parentheses indicate number of larvae deformed.

³ Abnormalities of the median tooth (normally trifid) and first lateral tooth (normally bifid) include fusion of processes or extra processes.

* Cleft median tooth common in Control 1.

** Symmetrical partial fusion of processes on the first lateral common in Control 2.

TABLE 6: REPRODUCTIVE SUCCESS OF MALE CHIRONOMIDS FROM EMS-EXPOSED CULTURES

EMS Concentration (mg/L)	No. of Matings of Exposed Males to Single Females	Females Exposed/ Unexposed	No. of Egg Masses Obtained	No. of Egg Masses Viable
0	4	unexposed	4	4
50	2	exposed	2	2
75	5	unexposed	1	0
100	2	unexposed	2	0

FIGURE I

Normal Morphology of the
Hypostomial Plate in *Chironomus*

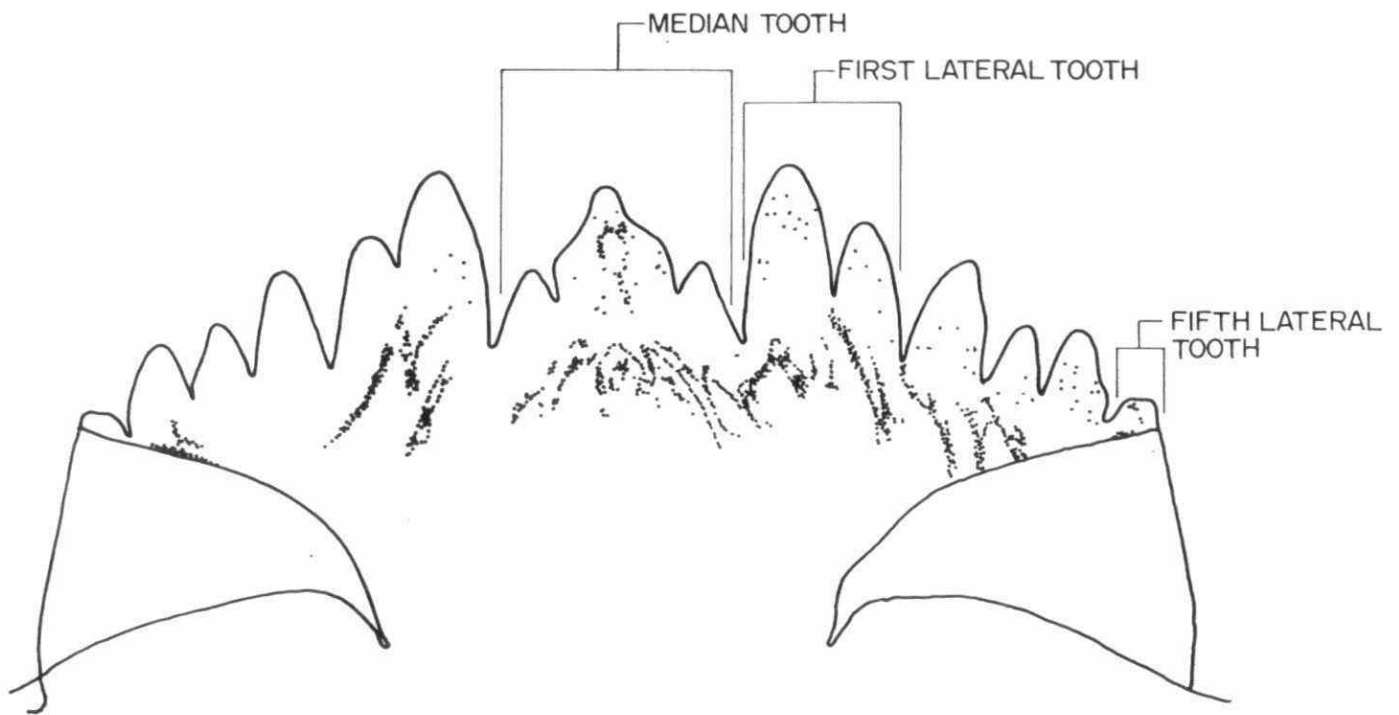


FIGURE 2 Relationship of Mouthpart Deformities to EMS Exposure

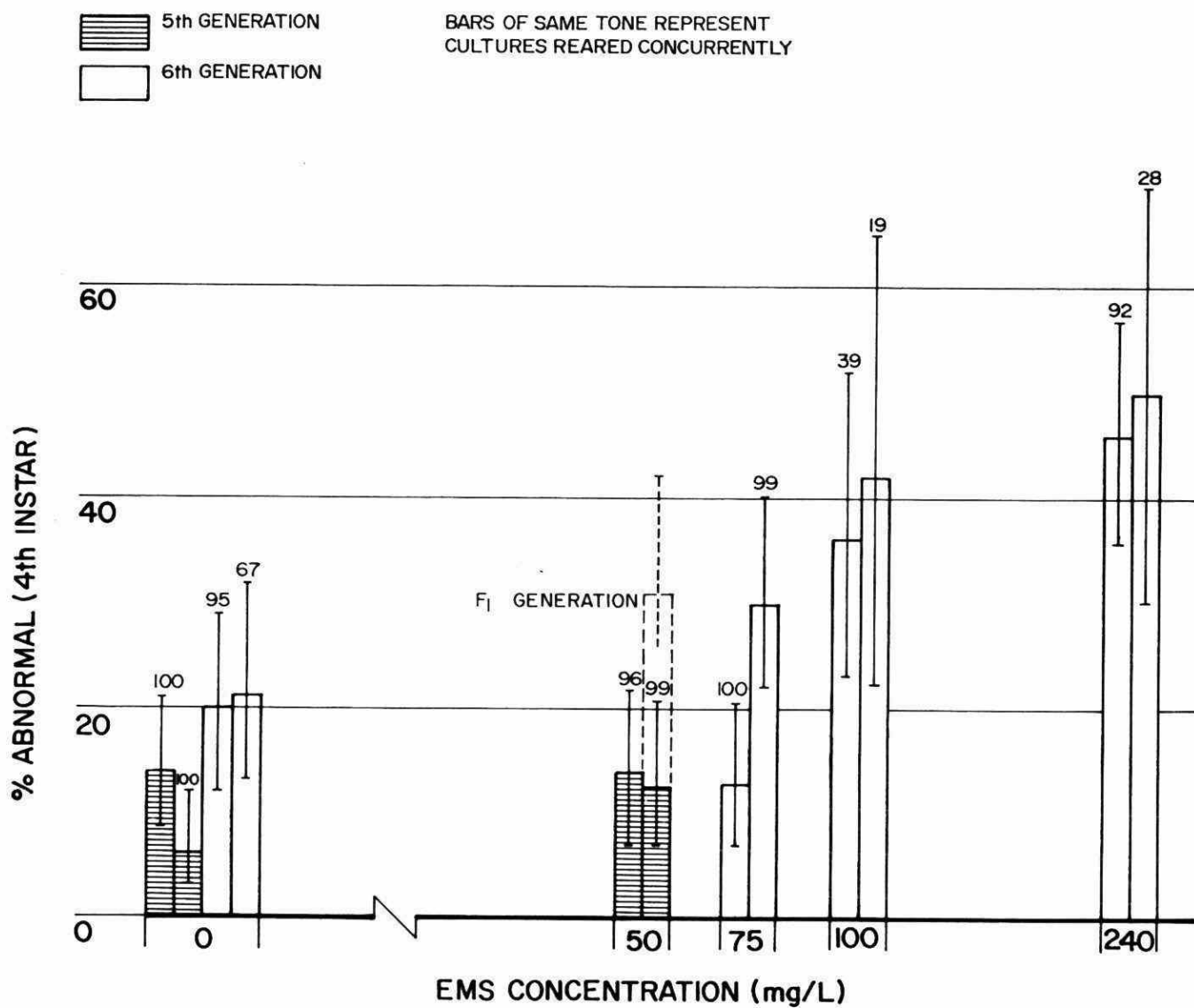
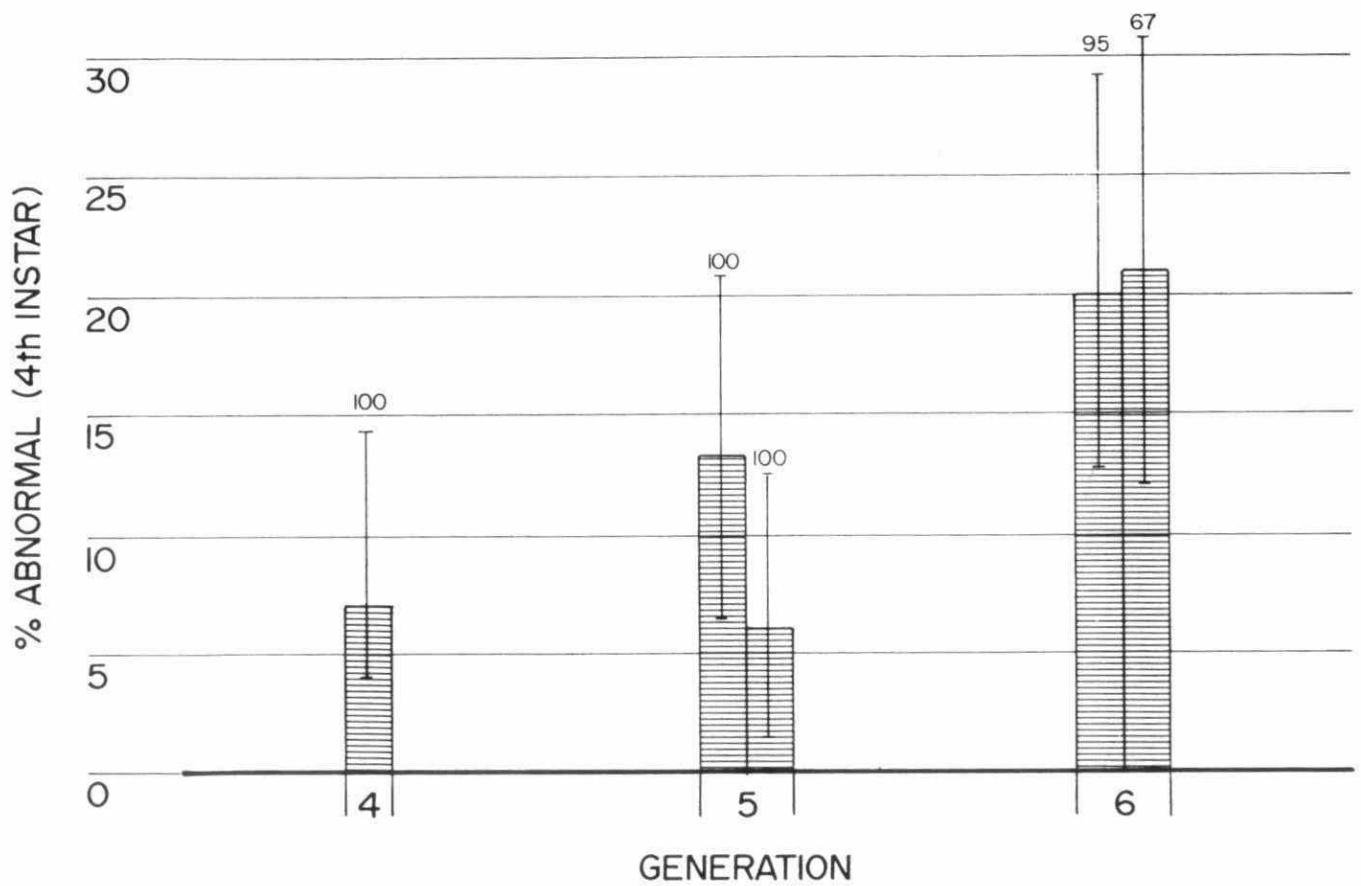


FIGURE 3 Control Deformity Incidence in Successive Laboratory Generations



Freshwater clams as monitors of variation in
environmental acidity and trace metal levels

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Summary

We collected freshwater clams (Elliptio complanata) from an acid sensitive and a circumneutral lake in south-central Ontario and compared tissue metal concentrations. Clams from the circumneutral lake had greater concentrations of Zn and Mn and lower concentrations of Cu and Cd than clams from the acid sensitive lake. Clam size and/or age successfully predicted tissue metal levels, suggesting that long-term clam biomonitoring studies should control for size and age variability.

We examined the effects of environmental acidity on E. complanata shell morphology and concluded that differences in shell size and shape were a function of within-lake variation in water turbulence. Therefore the use of shell morphology as an indicator of environmental acidification requires prior consideration of within-lake variability in shell form.

Clam shells were not useful monitors of environmental trace metal levels.

Introduction

Molluscs have, for over 10 years, been used to monitor contaminant levels in the marine environment (Goldberg et al. 1978). They accumulate and concentrate a wide range of environmentally available pollutants, and they are widely distributed both globally and in terms of the habitats that they occupy (Green et al. 1985). In recent years, freshwater clams have been used to monitor variation in environmental contaminant levels through in situ (Forester 1980; Imlay 1982; G.L.I. 1984; Pugsley et al. 1985) and transplantation (Kauss et al. 1981; G.L.I. 1985) studies. It is uncertain, however, whether freshwater clams exhibit size- and age-specific contaminant accumulation patterns similar to their marine counterparts (Boyden 1977; Romeril 1979; Cossa et al. 1980). This must be determined before freshwater clams are extensively used as biomonitors. In this study we examine size- and age-specific patterns of trace metal concentrations in the freshwater clam Elliptio complanata.

The bioavailability of many trace metals is enhanced by increasing environmental acidity (Campbell et al. 1983; Campbell and Stokes 1985; Stokes et al. 1985; Kelso et al. 1986). In acid stressed, poorly buffered waters, bioaccumulation of trace metals has been reported in clams (Forester 1980; Graney et al. 1984). We will compare tissue metal concentrations in E. complanata from an acid sensitive and a circumneutral lake. The relative importance of these patterns will be examined by comparing them to the size- and age-specific relationships.

Although clam tissues are useful for monitoring short-term fluctuations in aquatic contaminant levels, clam shells may be useful as indicators of long-term water quality patterns. It has been suggested that variation in environmental acidity can cause variation in shell shape (Mackie and Flippance 1983) and growth rate (Green 1980; Singer 1981) because calcium, which is necessary for shell growth, is often less available in low alkalinity environments. We examined this hypothesis by sampling E. complanata from four small lakes that vary in alkalinity and compared within-lake to between-lake variation in shell morphology.

Marine clam shells can retain a record of contaminant levels over many years (Fang and Shen 1984), so clam shells may provide a more long-term and irreversible system for monitoring changes in environmental levels of trace metals than clam tissues. We utilized the electron probe and the secondary ion mass spectrometer to assess trace metal levels in thin sections of freshwater clam shells sampled from known contaminated areas.

Methods

Age- and size-specific trace metal patterns

We collected 50 E. complanata, including as wide a size range as possible, from the southwest corners of Beech Lake and Tock Lake using SCUBA. These lakes are located in south-central Ontario on the Precambrian Shield (Fig. 1). Although morphologically similar, the lakes vary greatly in alkalinity

with total inflection point alkalinities for Tock and Beech of 22 and 153 ueq/l respectively. Shell lengths were determined and used as a measure of clam size. Clam tissues were dissected into gill and remaining visceral mass (body), then dried and digested in concentrated nitric acid, and analyzed using flame spectrophotometry for levels of Cu, Cd, Zn, and Mn. Shells were aged by the thin-section method (Clark 1980).

Analysis of variance (ANOVA) was used to compare differences in gill and body trace metal concentrations between the lakes. Analysis of covariance (ANCOVA) was used to compare the relationships between clam age and length and tissue metal concentration. Tissue metal concentration and length data were log-transformed, prior to analysis, to reduce error variance heterogeneity.

Within- and between-lake variation in shell form

We collected 25 E. complanata using SCUBA from a low (southwest) and a high (northeast) exposure site (i.e. water turbulence) in each of four small lakes (Figs. 1 and 2). Only old individuals were collected to eliminate age structure as a confounding factor. Shell length, height, width and weight were determined (Fig. 3). These data were log-transformed to linearize allometric relationships between the variables.

A model II ANOVA estimated the proportion of the total variance of each variable due to variation between the four lakes, between exposure levels within the lakes and between individual clams within the sites.

Shell analysis for trace metals

Thin sections of clam shells were prepared for the electron probe following the methods of Lutz (1981). As, Hg, Pb, Cd, Ca, Sb and Zn levels can be determined by the characteristic x-rays which are emitted from the thin section when an electron beam is focussed on it. The secondary ion mass spectrometer, which is a more sensitive instrument than the electron probe, was also used to determine levels of the same metals in the thin sections.

Results

Age- and size-specific trace metal patterns

Trace metal concentrations in both gill and body tissues differed between Beech Lake and Tock Lake clams (Table 1). Tock Lake clam tissues were higher in Cu and Cd and lower in Zn and Mn than Beech Lake clam tissues ($P < 0.05$). There was not any strong relationship between clam size and age possibly because of the lack of extremely small individuals in the sample. Size and age could therefore be used as independent covariates in each analysis. Each model (Table 2) was successful in predicting tissue trace metal variability from clam size and age.

Within- and between-lake variation in shell form

In Green, Beech, Bark and Tock Lakes, clams from the high exposure site had longer, taller, wider and heavier shells than those from the low exposure site (Table 3). Components of variance computed from the model II ANOVAs revealed that much

more of the variation in shell morphology resulted from differences between exposures rather than differences between lakes (Fig. 4).

Shell analysis for trace metals

The electron probe analyses showed no detectable levels of As, Hg, Cd, Pb or Zn in the shells examined. The secondary ion mass spectrometer detected only major shell elements (i.e. Ca, C, Sr, Na, Fe, Mn) which were in part per thousand concentrations.

Discussion

Age- and size-specific trace metal patterns

Cu, Cd, Zn and Mn are known to increase in geochemical mobility with increasing acidification (Campbell et al. 1983) and are generally in greater concentrations in Ontario non-buffered versus buffered waters (Forester 1980). Tock Lake is acid sensitive and has low buffering capabilities while Beech Lake is circumneutral and thus possesses the ability to buffer fluctuating levels of acidic input. Analyses of a small number of water samples from these lakes (unpub. data) suggest that absolute levels are generally greater in Tock Lake. However, Beech Lake has greater Mn and Zn levels relative to Cu and Cd than does Tock Lake. Concentrations of metals in the clams showed similar patterns, as Beech Lake clams had higher Mn/Cu, Mn/Cd, Zn/Cu and Zn/Cd ratios. These results closely resemble

those of Servos (pers. comm.; Freshwater Institute, Winnipeg Manitoba) who examined bioaccumulation of metals by E. complanata during acidic snowmelt. In his study, Zn concentrations increased relative to Cd concentrations in gill and body tissues as environmental acidity decreased.

There can be competition between metals for a limited supply of ligand (binding substrate) within marine mollusc tissue, and the competitive nature of metals for ligands can be altered depending on environmental metal levels (Mason and Simkiss 1983). It is thus possible that higher absolute metal levels (i.e. in Tock Lake) enhance the competitive nature of Cu and Cd for binding substrate while lowering that of Zn and Mn. Competition between trace metals for binding substrate in E. complanata tissues has been reported by Tessier et al. (1984) who determined that Fe competes with Cu and Zn. They suggested that Fe plays a protective role as it is the principal factor controlling the dissolved trace metal concentrations to which the clam is exposed.

Gill tissue metal concentrations were often twice that of body tissue. Gill concentrations may be overestimated and may not reflect biologically available metals, because available and non-available metals can adhere to mucous covered gill surfaces. Including gills in whole body analyses may therefore be inappropriate, because they could decrease the predictive power of the model by increasing body tissue trace metal variability.

Size- and age-specific trace metal relationships were different for each tissue type and each metal. This type of variability is common in marine bivalves (Boyden 1977; Romeril

1979; Cossa et al. 1980; Popham and D'Auria 1983). There are many factors which could enhance variability in these patterns such as feeding habits, reproductive state and condition factors. However, physiological explanations for the observed patterns are not within the scope of this paper. Size and age are important factors which contribute to tissue trace metal variability. Therefore, investigators who wish to compare E. complanata metal concentrations should standardize clam size and age. This would especially apply to clam biomonitoring studies where comparisons are made among sites and years.

In only two cases (gill Cu and body Cd) did lake interact with clam size and/or age in modelling tissue trace metal variability. Buffering capability, which is the primary difference between the two lakes, is therefore not very important in influencing size- and age-specific patterns. However, with only two lakes in the analyses, we have very little power to detect such an influence.

Within- and between-lake variation in shell form

Exposure affected variation in shell morphology similarly in all four lakes. Clams from low exposure sites were smaller and lighter than those from high exposure sites. These characteristics may be adaptive because small, light shells would preclude sinking in low exposure, soft silt areas while large, heavy shells would prevent dislodgement by wave action in high exposure, sandy areas.

Some researchers have suggested that variation in alkalinity

can cause variation in clam growth patterns (Green 1980; Singer 1981; Mackie and Flippance 1983). However, interpretation of those studies is often difficult because within-lake variation in growth patterns was not considered. In this study, much more of the total variation in shell form was due to within-lake between-exposure differences rather than between-lake differences. The primary difference between the four lakes is alkalinity; therefore alkalinity has little to do with variation in E. complanata shell form. Our results indicate that within-lake variation in shell form must first be assessed or controlled by the study design, before among-lake variation is examined.

Shell analysis for trace metals

The electron probe has a maximum sensitivity of 200 ppm, however, it could not detect As, Hg, Cd, Pb or Zn in the shells examined. Similar results were obtained from the more sensitive secondary ion mass spectrometer. Body tissue analyses of the same specimens had shown significant As, Hg and Pb concentrations (Metcalf and Mudrock 1985).

Conclusions

Freshwater clam tissues can be a useful tool for monitoring in situ levels of biologically available trace metals in areas affected by acidic precipitation once size and age effects are controlled for. Before large scale transplantation studies are designed, heritability of trace metal uptake rates should be examined. For example, Hinch et al. (1986) concluded that growth

rates of the unionid clam Lampsilis radiata were a function of the population source (in a field transplant experiment). Other physiological processes which may be related to trace metal uptake may also be a function of the population source. A three-way reciprocal transplant experiment has been conducted using E. complanata and three lakes which represent the "degree of acidification" gradient. These data are presently being analysed. Electrophoretic studies in allozyme frequencies are being done, to investigate the role of genotype in trace metal uptake.

Although clam shells have proven to be useful monitors of contaminant levels in marine environments, they were not useful for monitoring levels of trace metals at the environmental concentrations observed in this study. Shell morphology is not a good predictor of environmental acidity but probably reflects site-specific water turbulence.

Acknowledgements

Linda Stephenson and Vicki Young prepared the clam tissues for metal analysis and the shells for aging. Cindy Walker prepared and analyzed shell sections for trace metals. Robert Bailey commented on earlier drafts of this paper. The Ontario Ministries of the Environment and Natural Resources, and Environment Canada provided important data for this study. This study was made possible through NSERC and Ontario Ministry of the Environment operating grants to R. H. Green, and an Ontario Graduate Scholarship awarded to S.G. Hinch.

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the Knysna estuary. Marine Pollution Bulletin, 7, 45-48.

Table 1. Mean metal concentrations in p.p.m (SD) in gill and body tissues of E. complanata from Beech Lake and Tock Lake. Each Mn concentration is x 102.

		Beech Lake		Tock Lake	
gill	Cu	13.28	(5.69)	18.51	(7.83)
	Cd	10.10	(4.34)	12.78	(4.49)
	Zn	371.32	(108.75)	273.81	(72.80)
	Mn	140.80	(40.30)	104.90	(32.70)
body	Cu	7.29	(1.83)	10.29	(6.95)
	Cd	10.98	(5.82)	14.51	(4.62)
	Zn	155.50	(43.87)	134.17	(63.15)
	Mn	42.20	(19.60)	23.50	(12.00)

Table 2. ANCOVA models which predict tissue trace metal variability from clam size and age in Beech Lake and Bark Lake.

dependent variable: gill Cu
model $r^2=.34$

<u>source of variation</u>	<u>P</u>
length	.106
age	.001
lake	<.001
length * age	.521
length * lake	.250
age * lake	.462
length * age * lake	.014

dependent variable: gill Cd
model $r^2=.18$

<u>source of variation</u>	<u>P</u>
length	.002
age	.443
lake	.021
length * age	.457
length * lake	.737
age * lake	.170
length * age * lake	.456

dependent variable: gill Zn
model $r^2=.25$

<u>source of variation</u>	<u>P</u>
length	.759
age	.003
lake	<.001
length * age	.642
length * lake	.378
age * lake	.278
length * age * lake	.403

dependent variable: gill Mn
model $r^2=.23$

<u>source of variation</u>	<u>P</u>
length	.923
age	<.001
lake	<.001
length * age	.717
length * lake	.634
age * lake	.303
length * age * lake	.692

dependent variable: body Cu
model $r^2=.20$

<u>source of variation</u>	<u>P</u>
length	.049
age	.073
lake	.004
length * age	.043
length * lake	.532
age * lake	.885
length * age * lake	.241

dependent variable: body Cd
model $r^2=.50$

<u>source of variation</u>	<u>P</u>
length	<.001
age	.300
lake	<.001
length * age	.033
length * lake	.015
age * lake	.602
length * age * lake	.887

dependent variable: body Zn
model $r^2=.20$

<u>source of variation</u>	<u>P</u>
length	.035
age	<.001
lake	.005
length * age	.739
length * lake	.598
age * lake	.952
length * age * lake	.727

dependent variable: body Mn
model $r^2=.42$

<u>source of variation</u>	<u>P</u>
length	.119
age	<.001
lake	<.001
length * age	.911
length * lake	.549
age * lake	.261
length * age * lake	.406

Table 3. Means (SD) of shell characters from low and high exposure sites in the study lakes. In all groups n=25.

Site	Length (mm)	Height (mm)	Width (mm)	Weight (g)
Tock low	66.0 (5.4)	33.2 (2.7)	17.7 (1.9)	9.6 (2.9)
Tock high	74.1 (5.8)	37.2 (3.0)	19.6 (2.1)	14.1 (3.6)
Bark low	59.4 (4.5)	28.6 (1.8)	17.8 (1.5)	8.1 (2.0)
Bark high	64.2 (3.1)	30.9 (1.5)	19.4 (1.2)	10.8 (2.1)
Beech low	63.3 (6.3)	31.6 (3.5)	16.7 (2.4)	8.5 (2.8)
Beech high	72.1 (6.3)	36.6 (3.1)	19.7 (2.2)	13.6 (3.7)
Green low	63.0 (8.6)	31.1 (4.2)	16.2 (2.6)	8.8 (4.5)
Green high	66.9 (7.7)	33.1 (3.8)	17.7 (2.5)	11.0 (4.7)

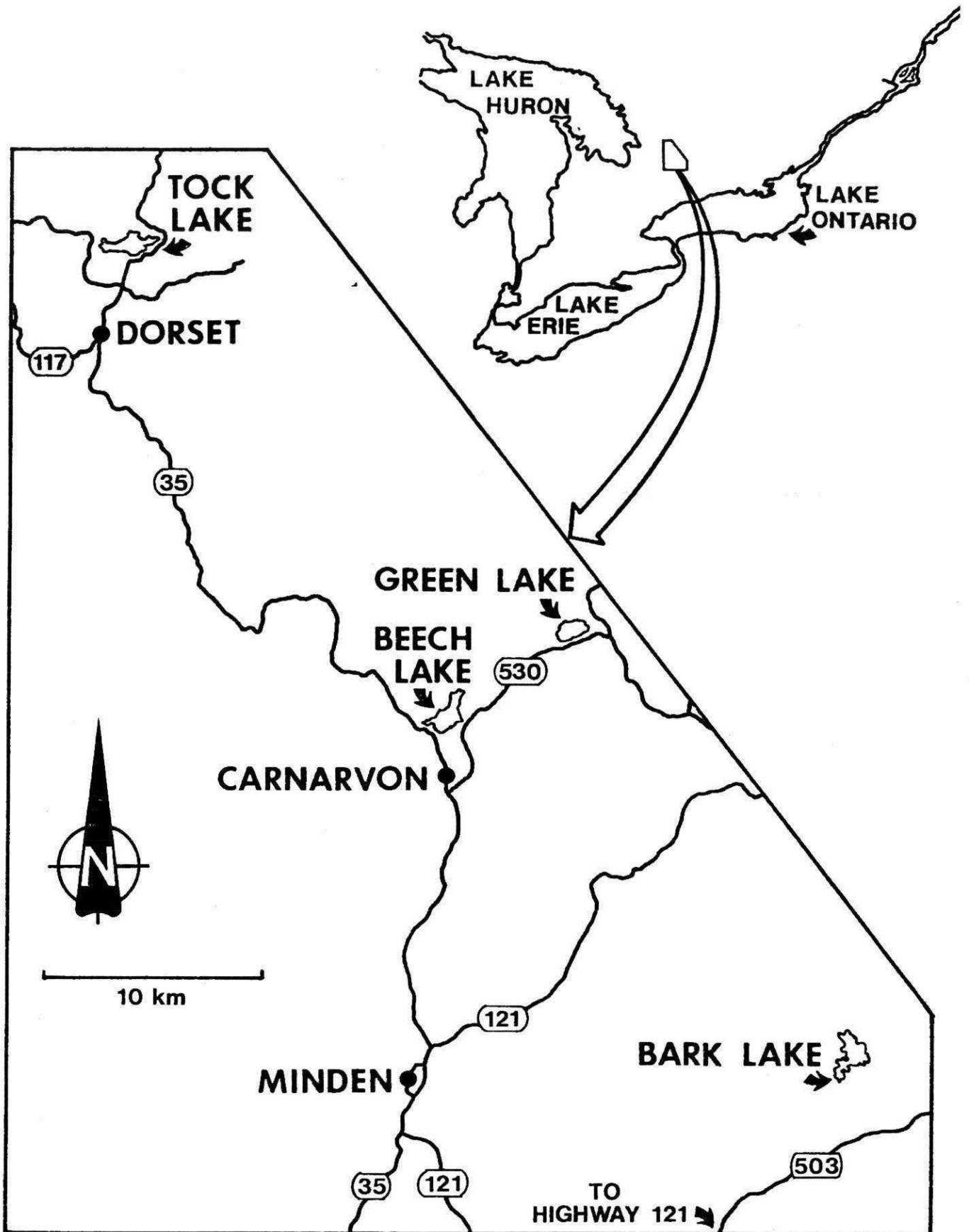
List of Figures

Fig. 1. Location of study area in south-central Ontario and detailed locations of lakes in relation to provincial highways and towns.

Fig. 2. Four study lakes with the low (L) and high (H) exposure sites in each lake.

Fig. 3. A drawing of Elliptio complanata (Unionidae) showing length, height and width dimensions.

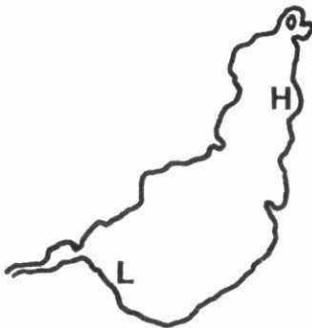
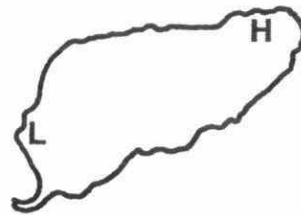
Fig. 4. Proportions of total variance in shell height, length, weight and width due to variation among the four lakes, between exposure levels within the lakes and among individual clams within the sites.



TOCK LAKE



GREEN LAKE



BEECH LAKE

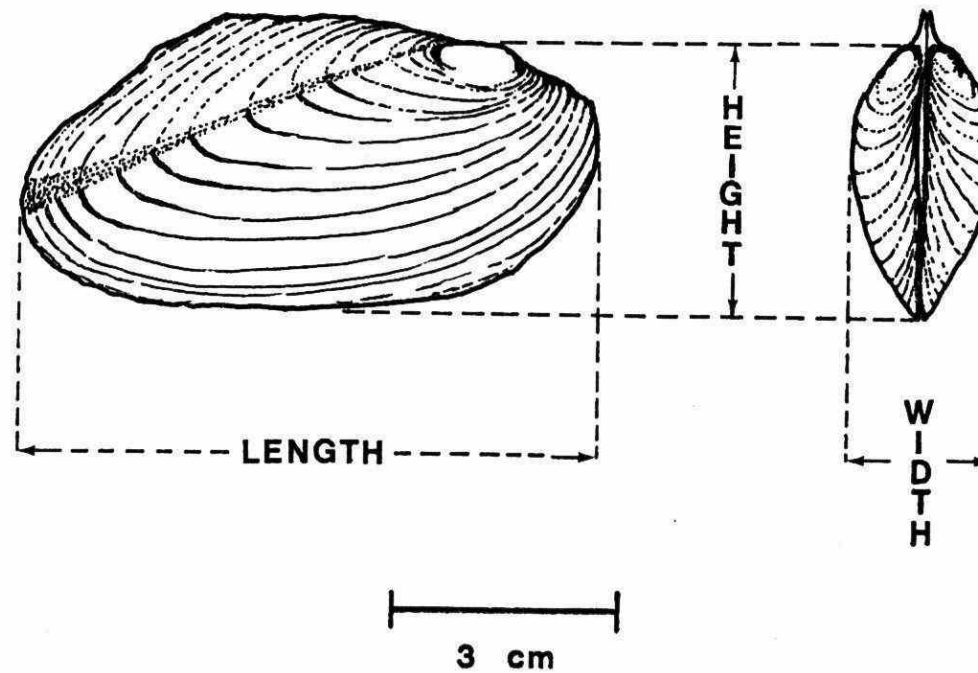


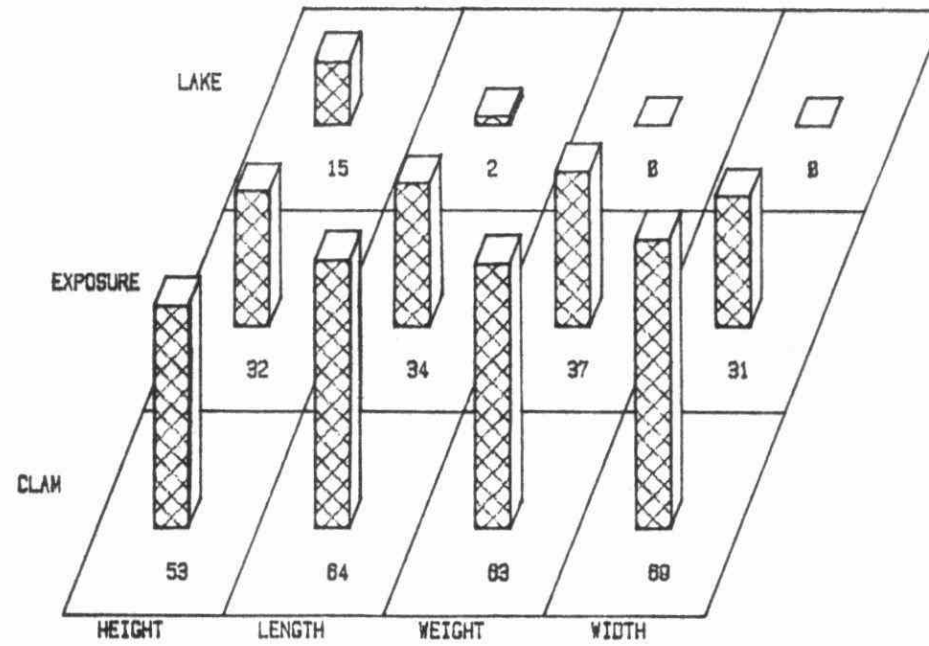
BARK LAKE

2 km

A horizontal line with vertical end caps, representing a scale bar for 2 km.

Elliptio complanata





ANALYSIS OF DIOXINS AND FURANS IN FISH AND OTHER BIOTA

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INTRODUCTION

The method currently used by the Ontario Ministry of the Environment for the determination of polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF) is based upon the procedure developed by Lamparski, Nestricks, and Stehl (1). The Dow procedure involves the use of multiple open-column chromatographic cleanup steps followed by fractionation on reverse phase HPLC in order to yield a sample suitable for analysis by GC/MS techniques. The analytical scheme employed by the Ministry of the Environment is outlined in figure 1. This procedure is tedious as well as time consuming. The cleanup method has been found to be ineffective for very complex samples, particularly for those containing high levels of polychlorinated biphenyls (PCB). In addition it is not possible to simultaneously determine other environmental pollutants because of poor recoveries or improper fractionation.

This study involves the development of a new cleanup procedure for the analysis of PCDD and PCDF in fish tissue. Work was carried out to devise an HPLC fractionation which would isolate the PCDD and PCDF from potential interferences found in biological samples such as fish. Fractionation of the sample by HPLC rather than open-column chromatography has several advantages. Preparation of chromatographic columns is tedious and consumes large amounts of both time and materials. HPLC allows for rapid and much more reproducible fractionation of sample components. The potential of automated sample injection, data acquisition, and fraction collection makes HPLC techniques even

more attractive.

The HPLC method developed to replace the column chromatography cleanup consists of a two-step fractionation procedure. The PCDD and PCDF are initially isolated from the bulk of the sample matrix using normal phase HPLC. A second fractionation is then carried out on reverse phase HPLC to further isolate the PCDD and PCDF from any interferences which were coeluted during the normal phase separation. Additional fractions may be collected in the first fractionation step if other sample components are to be analyzed. The overall analytical scheme for the analysis of PCDD and PCDF in fish is represented in figure 2.

EXPERIMENTAL

Instrumentation:

All HPLC fractionations were carried out using a Varian Model 5000 liquid chromatograph equipped with a fixed wavelength (254 nm) ultraviolet detector. The chromatographic injection system consisted of a Rheodyne pneumatic valve with a 100 uL loop injector. A Vista chromatography data system was interfaced to the HPLC allowing both manipulation of HPLC parameters and raw data collection on floppy disk. Normal phase fractionations were obtained using a semi-preparative scale silica column (25 x 0.94 cm) containing 10 um particles. The gradient elution program employed in the normal phase separation is shown in figure 3. A Micro-Pak MCH-10 ODS reverse phase column (30 x 0.94 cm, 10 um particles) was used for the second fractionation step. The reverse phase gradient elution program is illustrated in figure 4.

A Hewlett-Packard HP5987A GC/MS with an HP1000 data system and HP7914 disk drive was used for mass spectral analysis. An HP5880A gas chromatograph is directly interfaced to the quadrupole mass spectrometer by a capillary inlet. The chromatographic column used was a 30 meter DB-5 fused silica capillary column (0.25 mm i.d.) having a stationary film thickness of 0.25 μ m. All GC/MS analyses were made using a cool on-column injector to minimize discrimination against compounds with high boiling points and also provide efficient sample loading onto the column.

Chemicals and Solvents:

Organic solvents used in this study were hexane, benzene, dichloromethane, and methanol. All solvents were distilled in glass and are suitable for pesticide analysis (Caledon Laboratories Ltd., Georgetown, Ontario). Water for the reverse phase HPLC fractionation was distilled using a Kontes still (Vineland, New Jersey).

Reagent grade anhydrous sodium sulphate (J.T. Baker Chemical Co., Phillipsburg, New Jersey) was washed with 50 mL of hexane prior to use but was otherwise used in the form obtained by the supplier.

Dibenzodioxin and dibenzofuran standards were supplied by the Ministry of the Environment and also obtained directly from Cambridge Isotopes Laboratories (Woburn, Massachusetts). Analytical standards for other pollutants such as PCB, chlorinated pesticides, and polycyclic aromatic hydrocarbons (PAH) were obtained from a variety of sources.

Glassware Cleaning:

All glassware was cleaned by ultrasonic agitation in a water bath containing a mild detergent. Individual pieces were then thoroughly rinsed with deionized water and dried at 250°C overnight. All glass apparatus was rinsed three times with an appropriate organic solvent immediately prior to use.

HPLC Mobile Phase Preparation:

Solvents employed as HPLC mobile phases were filtered and degassed before use. Solvent filtration was achieved using a clarification kit consisting of a one-litre flask and 300 mL filter reservoir which is fitted with a replaceable 0.45 µm HVLP filter (Waters Millipore, Milford, Massachusetts). Following filtration each solvent was then deaerated by application of a vacuum and mechanical stirring.

Sample Preparation Prior to Normal Phase HPLC Fractionation:

Fish tissue extracts were obtained from the Ministry of the Environment and only required concentration prior to HPLC fractionation. Initial sample preparation carried out by Ministry personnel was as follows: Fish tissue is homogenized and then transferred to a clean, dry glass jar. Twenty grams of tissue is placed into an erlenmeyer flask to which 70 mL of concentrated hydrochloric acid is added. The tissue is allowed to digest at room temperature for at least 16 hours. The resulting mixture is extracted with three 50 mL aliquots of hexane. The combined organic extract is then concentrated to approximately 20 mL.

Before the fish tissue extract can be fractionated by HPLC, it is necessary to perform a preliminary cleanup step in which lipids and large polar molecules are removed. This is accomplished using a chromatographic column with sulphuric acid/silicic acid packing. This packing is made up by thoroughly mixing silicic acid (activated at 180°C) with sulphuric acid such that the resulting material is 44 percent H_2SO_4 by weight. Approximately 25 grams of the packing is placed in a glass column (100 x 10 mm i.d.) with a 100 mL reservoir. The column is pre-washed with 125 mL of hexane and then the sample is loaded onto the column with a disposable pipette. The desired organic components are eluted with 50 mL of hexane. The eluant is finally transferred to a clean glass vial where it is concentrated down to approximately 10 mL. The extracts were delivered in this form to our laboratory and the volumes were further reduced to 200 μ L before injection on the HPLC.

PCDD and PCDF retention behaviour on normal phase HPLC was established using a flyash extract since it is a convenient source of all of the various isomers (2). This extract was obtained by the Soxhlet extraction of approximately 40 grams of municipal incinerator flyash (Toronto, Ontario) with 300 mL of benzene for 24 hours. The extract was then concentrated down to about 1 mL and loaded onto a Sep-Pak silica cartridge (Waters Millipore, Milford, Massachusetts) which had been pre-wet with 50 mL of hexane. A complex mixture of compounds including the PCDD and PCDF are eluted with 50 mL of hexane while the more polar constituents are retained on the cartridge. The organic extract

is concentrated down to approximately 1 ml and transferred to a small glass vial. Finally the extract is concentrated to dryness under a gentle stream of high purity nitrogen (Union Carbide Canada Limited, Toronto, Ontario) and the residue is reconstituted with 200 uL of hexane.

Sample Preparation Prior to Reverse Phase HPLC Fractionation:

The components of all fractions collected during normal phase HPLC separation must be transferred into a solvent amenable to reverse phase HPLC before the second step fractionation can be carried out. This is accomplished by concentrating normal phase fractions to approximately 1 mL and transferring the extract to a glass vial where it is gently evaporated to dryness. The resulting residue is then redissolved in 200 uL of 10:90 benzene:methanol and is now ready for reverse phase HPLC.

Sample Preparation Prior to GC/MS Analysis:

The end-product of the two-step HPLC cleanup procedure is a solution containing the PCDD and PCDF along with numerous other compounds. The solvent matrix consists of a mixture of methanol and water (less than 10 percent) and therefore cannot be directly concentrated to yield a sample which can be chromatographed on the average capillary GC column. Therefore the PCDD and PCDF must be transferred to a solvent which is better suited to the application of GC techniques. This is done by extracting the reverse phase fractions with three 50 mL portions of hexane in a 250 mL separatory funnel. Each hexane extract is passed through a

sintered glass filter containing approximately 10 grams of anhydrous sodium sulphate (pre-washed with 50 mL of hexane) and collected in a round bottom flask. The combined extracts are then concentrated to approximately 1 mL and transferred to small glass vials. Each extract is then evaporated to dryness under a stream of high purity nitrogen and finally redissolved in 100 uL of benzene. The sample extracts are now ready for analysis by GC/MS.

RESULTS AND DISCUSSION

Initial development of HPLC fractionation methodology involved the use of several standards containing compounds characteristic of those found in fish tissue, including compounds such as PCDD, PCDF, PCB, PAH, and chlorinated pesticides. Preliminary optimization of the HPLC fractionations was accomplished based upon the comparison of the retention behaviour of two dioxins isomers with the other compound classes mentioned above. The 1,2,3,4-tetrachloro- and octachlorodibenzodioxin isomers (1,2,3,4-TCDD and OCDD respectively) were chosen to represent all PCDD and PCDF because they represent the extremes in degree of chlorination for the isomers normally determined. Also, sufficient amounts of each were available to create solutions concentrated enough to be detected by the UV detector of the HPLC. These solutions can later be used to verify the reproducibility of HPLC retention times to permit proper fraction collection times. Figures 5b and 6b show the HPLC traces of 1,2,3,4-TCDD and OCDD under the optimized gradient programs for normal and reverse phase HPLC respectively.

In order to confirm retention behaviour of PCDD and PCDF on both normal and reverse phase HPLC, a solution containing all of the different isomers must be injected on the HPLC and numerous fractions collected. These fractions are then analyzed by GC/MS using selected ion monitoring techniques. Four ions were monitored for each congener group to permit positive identification of the PCDD and PCDF. Since there was no standard available which contained all of the PCDD and PCDF isomers, a flyash extract was used. This extract contains an easily detectable amount of dioxins and furans with relatively few interfering compounds being present. Since the levels of PCDD and PCDF in fish tissue are often extremely low and in fact may be below instrumental detection limits, it is not possible to use tissue extracts to determine HPLC retention behaviour.

Since fish tissue often contains many compounds which interfere with the GC/MS determination of PCDD and PCDF, the effectiveness of the HPLC cleanup procedure had to be investigated. This was accomplished by determining the effect of introducing a potentially interfering matrix to flyash extract and comparing the reconstructed ion chromatograms of the fortified and nonfortified extract. Two fortified flyash extracts were prepared; the first extract was spiked with a relatively large amount of various Aroclor standards while the second was fortified with a typical fish tissue extract. The concentration of individual Aroclors in the fortified extract were: Aroclor 1016, 7 ug/uL; Aroclor 1242, 14 ug/uL; Aroclor 1254, 5 ug/uL; and Aroclor 1260, 8 ug/uL. The flyash extract and the two fortified

extracts were chromatographed under identical HPLC conditions and a single fraction containing the PCDD and PCDF was collected for each. The HPLC traces for the normal and reverse phase separations along with the fraction collection times are given in figures 7 and 8.

In the normal phase fractionation step, most of the PCB elute within the retention window of the PCDD and PCDF as can be seen by comparing parts a and b of figure 7. Therefore the first step fractionation procedure does not isolate the dioxins and furans from the potential interference from chlorinated biphenyls. However, the second step reverse phase fractionation does separate PCDD and PCDF from PCB. Figures 6b and 6c show that 1,2,3,4-TCDD and OCDD are well separated from the bulk of the PCB. A comparison of figures 8a and 8b reveals the effect of the addition of a large amount of PCB on the acquired separation. Clearly the majority of the chlorinated biphenyls are eluted quite early and the sections of the UV trace corresponding to the retention windows for PCDD and PCDF are virtually identical for the nonfortified and PCB-fortified flyash extract. Similarly the HPLC traces for the flyash and flyash-fish extracts (figure 8a and 8c respectively) are virtually identical with respect to the appearance of the traces within the fraction collection windows. The effectiveness of the separation obtained by employing the two-step HPLC cleanup procedure was confirmed by GC/MS. The reconstructed ion chromatograms for the individual congener groups of the PCDD and PCDF were found to be relatively free of interferences. Therefore the developed HPLC method appears to

provide a reasonable cleanup which will aid in the determination and dioxins and furans in fish tissue. This method should also be applicable to most other biological materials although the initial sample preparation procedure may require suitable modification.

References:

1. Lamparski, L.L.; Nestruck, T.J.; and Stehl, R.H.; Anal. Chem., 51(9), 1453-8, 1979.
2. Karasek, F.W. and Hutzinger, O.; Anal. Chem., 58(6), 633A-42A, 1986.

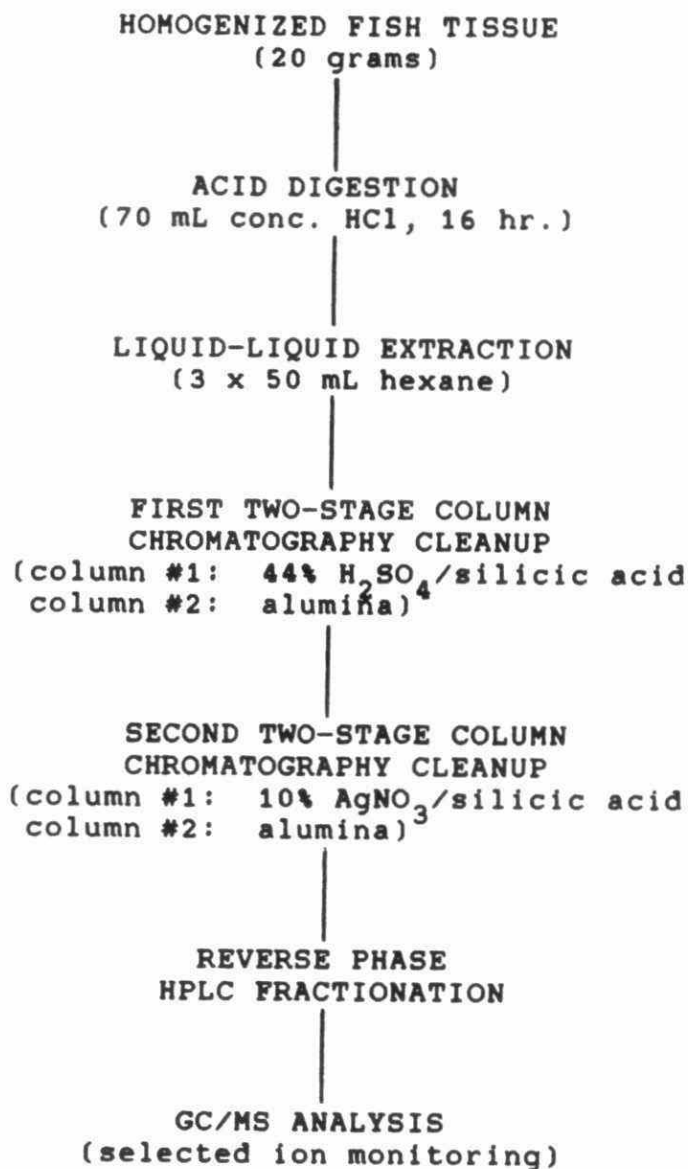


FIGURE 1: Current MOE procedure for the analysis of PCDD and PCDF in fish.

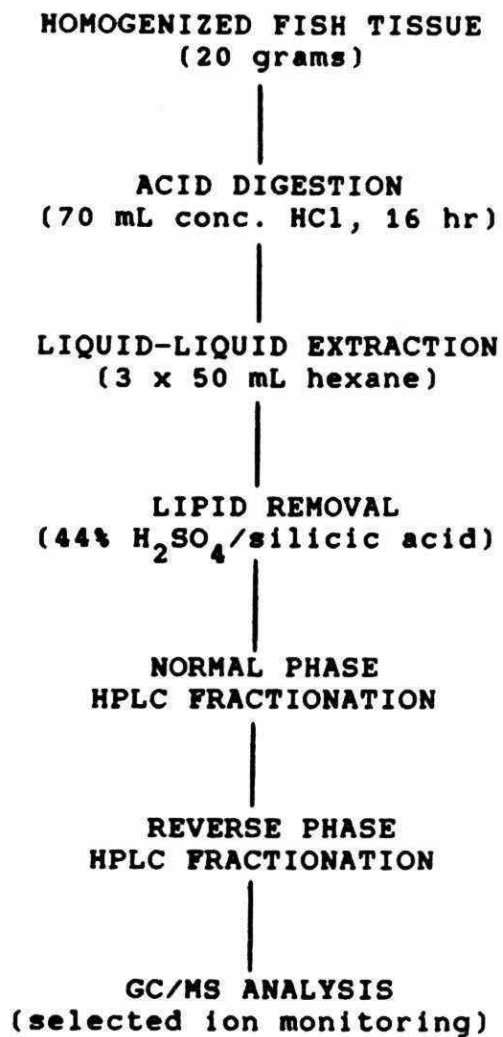


FIGURE 2: Proposed procedure for the analysis of PCDD and PCDF using a two-step HPLC fractionation.

Normal Phase HPLC Gradient Elution Program

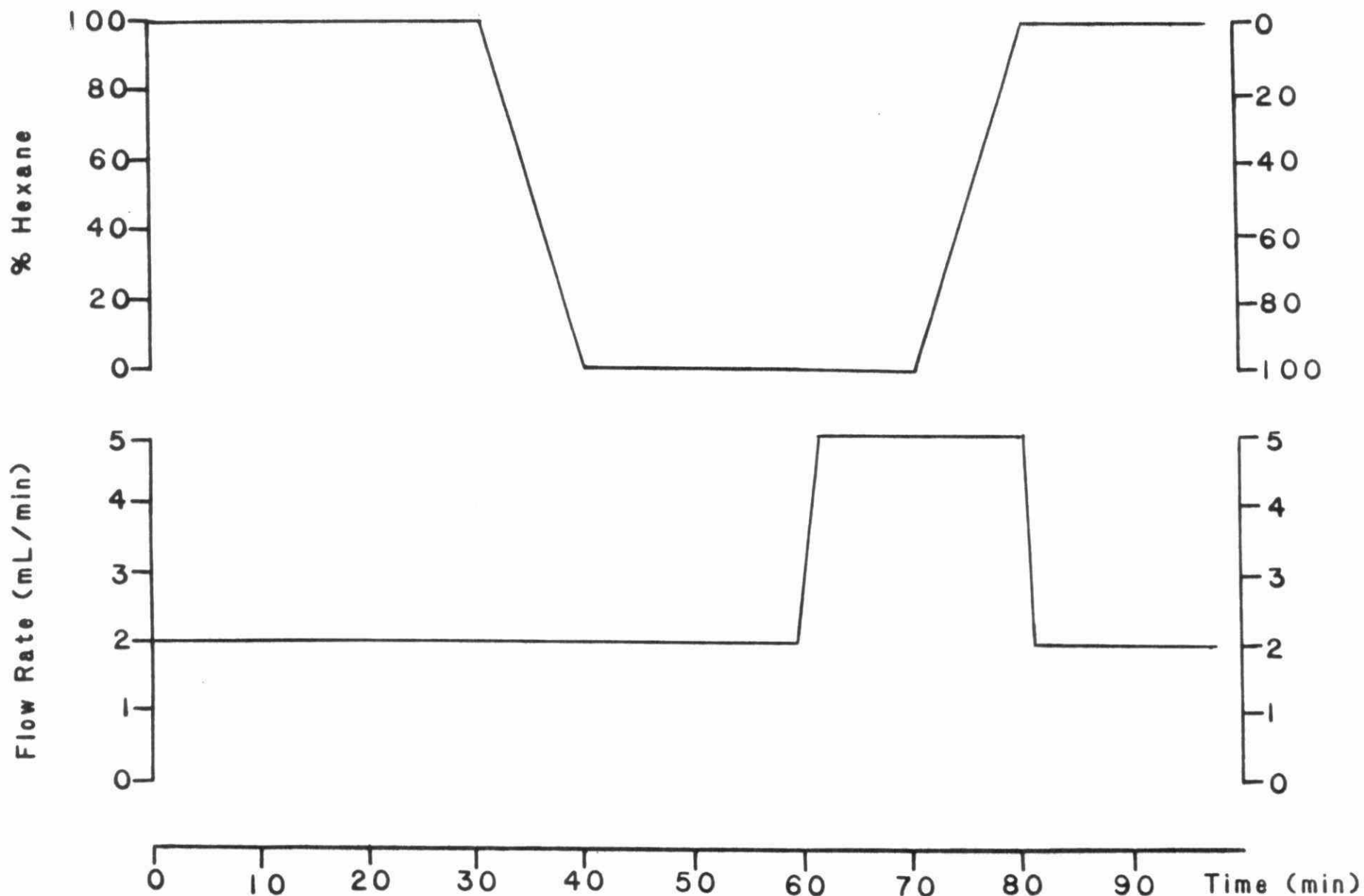


FIGURE 3: Mobile phase gradient elution program for normal phase HPLC.

% Dichloromethane

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Reverse Phase HPLC Gradient Elution Program

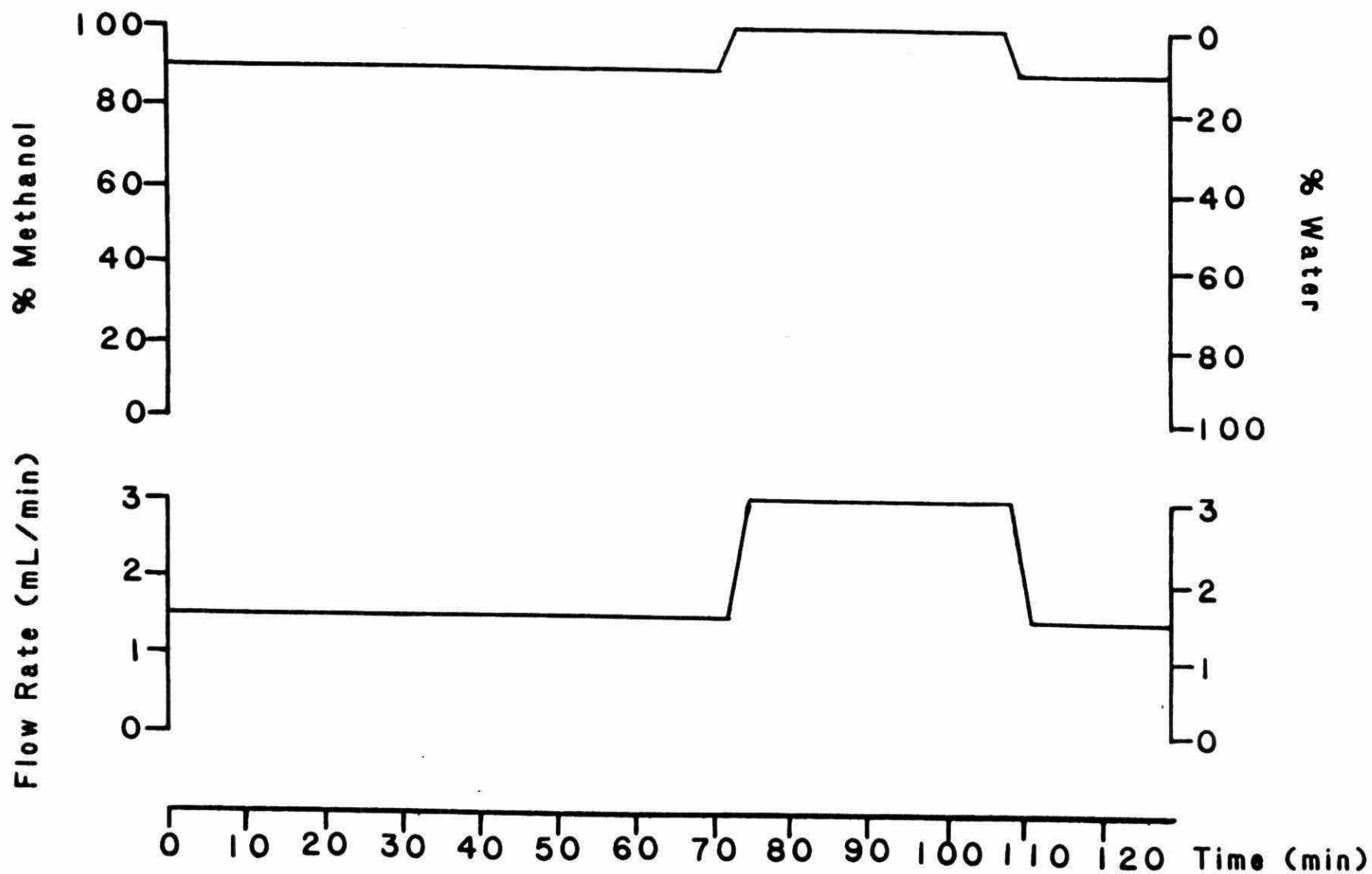


FIGURE 4: Mobile phase gradient elution program for reverse phase HPLC.

a)

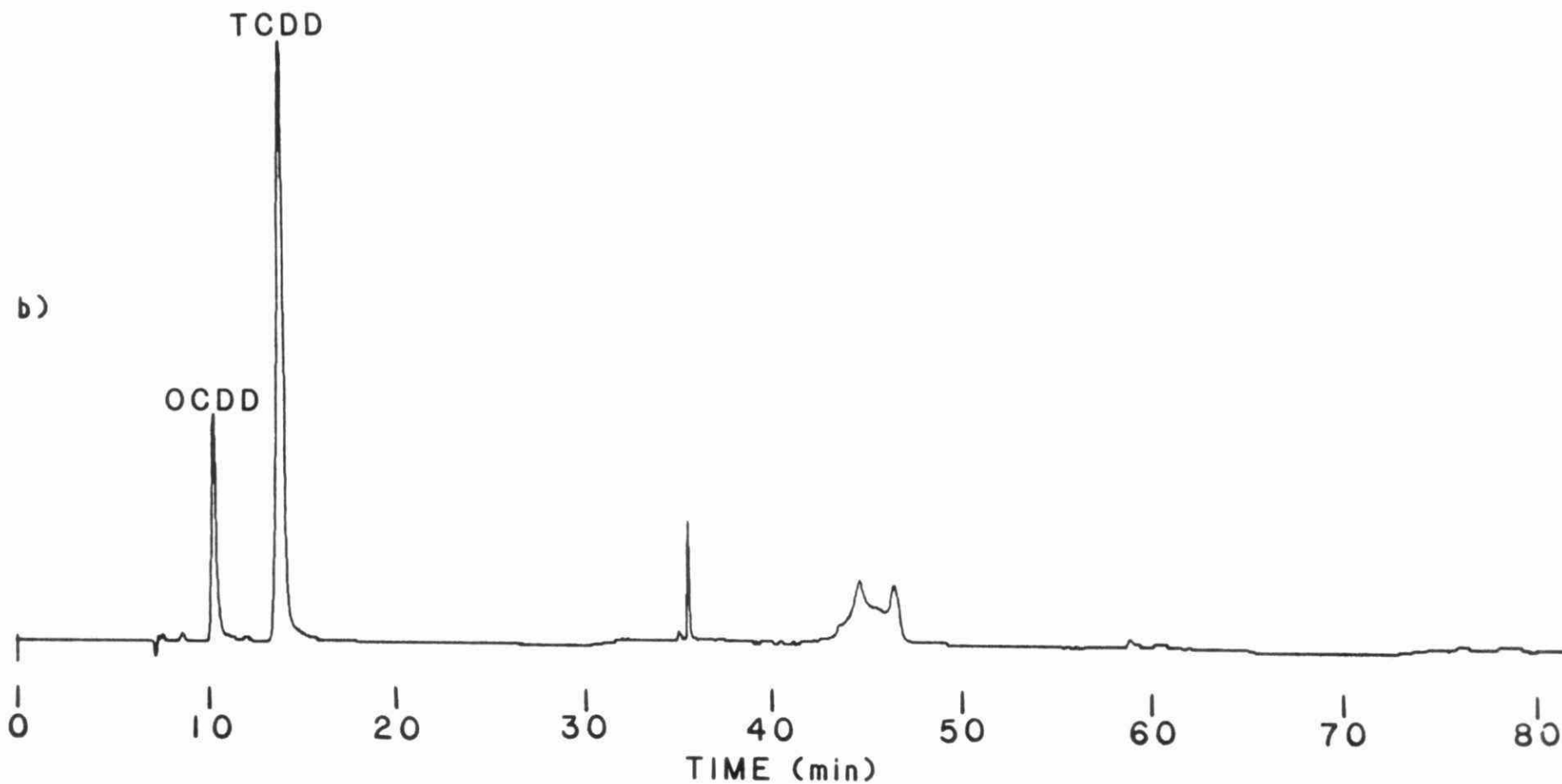
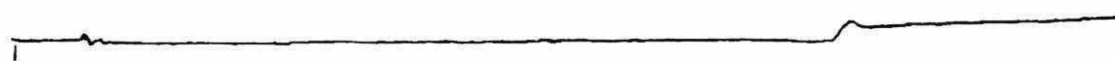
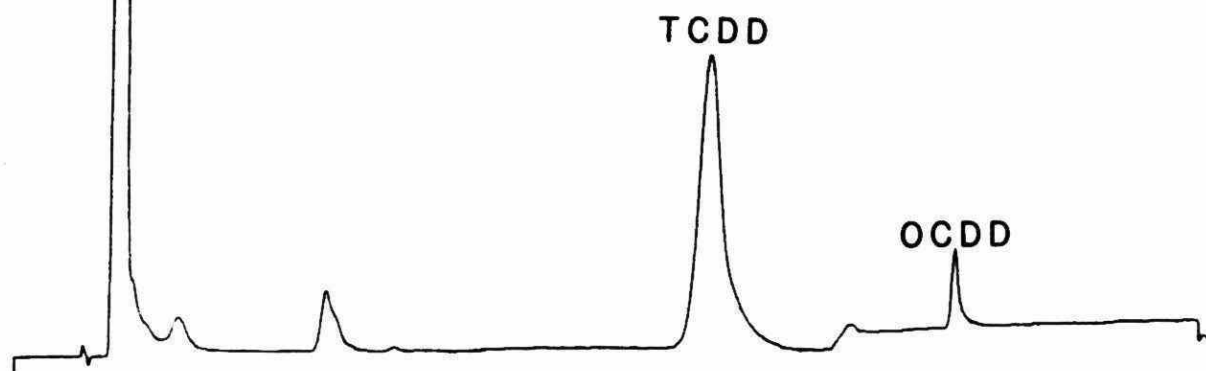


FIGURE 5: Normal phase-HPLC traces (UV detection) for a) solvent blank and b) 1,2,3,4-TCDD and OCDD.

a)



b)



c)

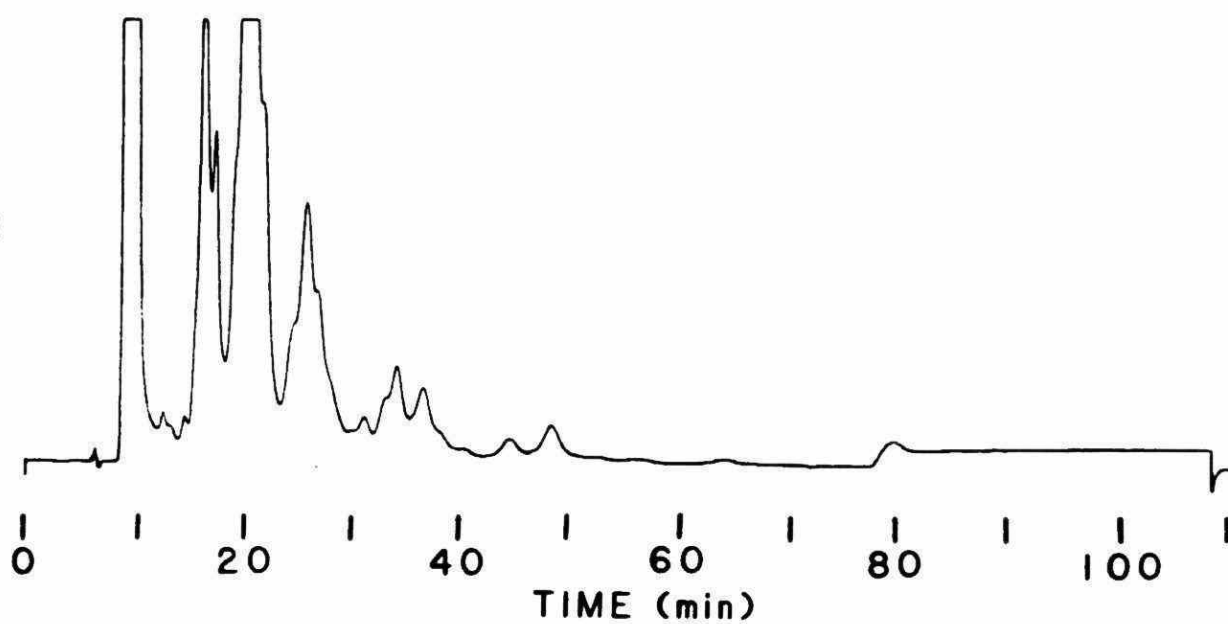


FIGURE 6: Reverse phase HPLC traces for a) solvent blank, b) 1,2,3,4-TCDD and OCDD, and c) PCB mixture (Aroclors 1016, 1242, 1254, and 1260).

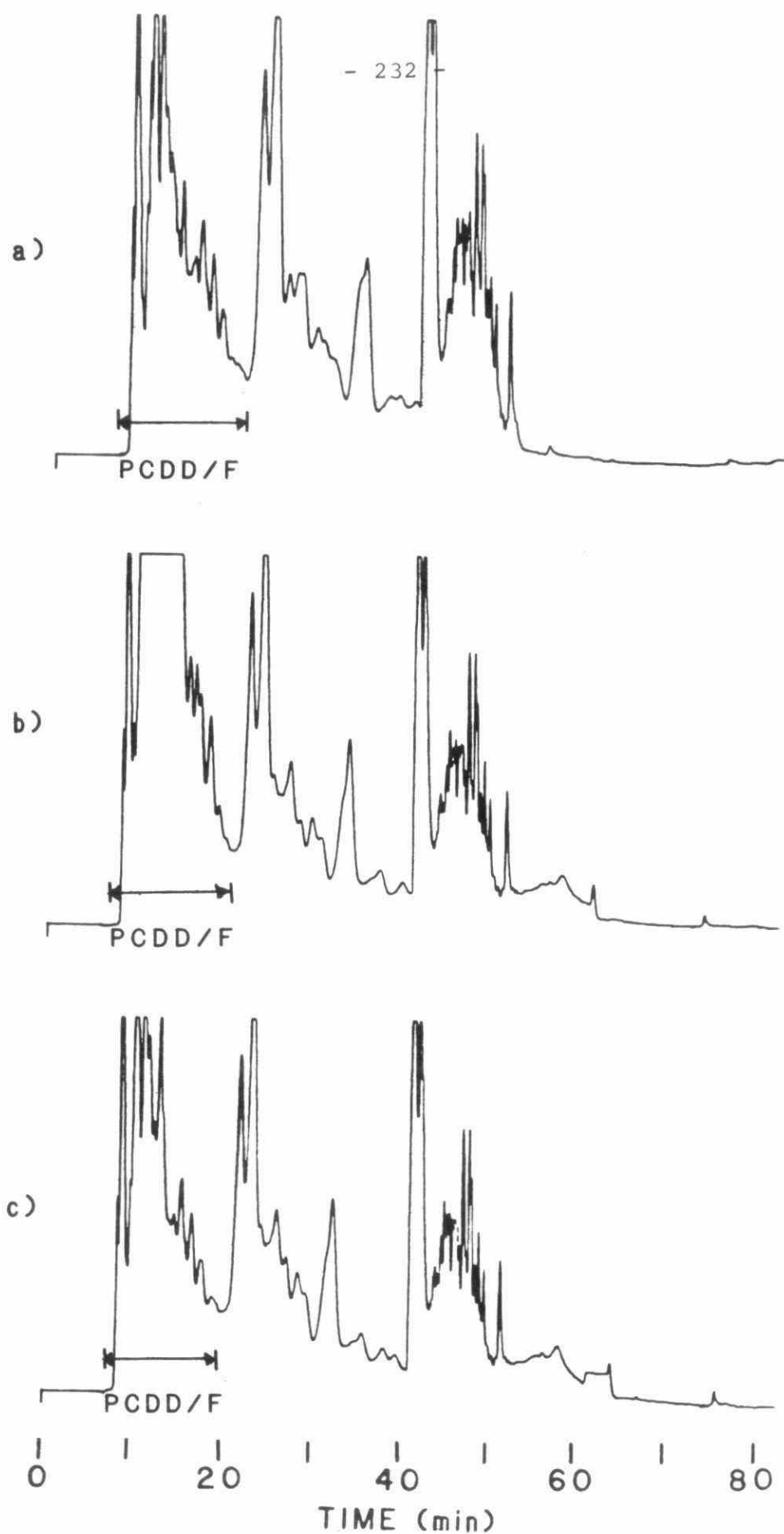


FIGURE 7: Normal phase HPLC traces (UV detection) of a) flyash extract, b) flyash extract fortified with PCB, and c) flyash extract fortified with fish tissue extract.

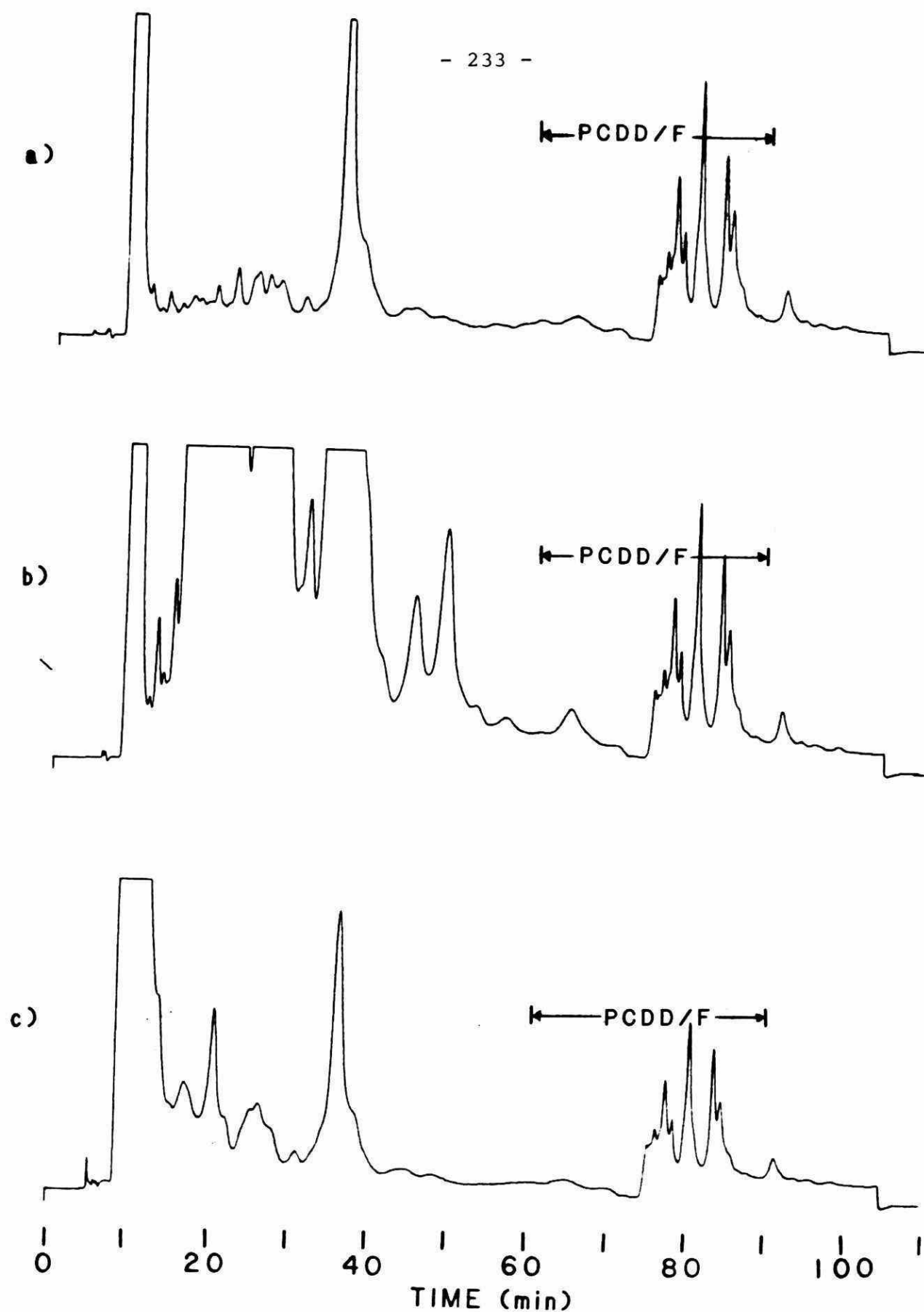


FIGURE 8: HPLC traces (UV detection) for the second-step fractionation of a) flyash extract, b) flyash extract fortified with PCB, and c) flyash extract fortified with fish tissue extract.

DEVELOPMENT OF A COST EFFECTIVE PROTOCOL FOR ROUTINE
ANALYSIS OF TRACE ORGANIC CONTAMINANTS IN MUNICIPAL WPCP RAW
SEWAGES AND FINAL EFFLUENTS

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and

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ONTARIO MINISTRY OF THE ENVIRONMENT

INTRODUCTION

The development of analytical methods for efficient processing of wastewater sample has always been a continual challenge. The vast array of organics present at low concentrations in municipal wastewater requires complicated extraction, isolation and detection procedures. Added to this is the fact that large amounts of interferences are usually present in the extracts, consequently requiring extensive cleanup procedures.

In 1979, the US EPA published a series of procedures for the analysis of the 129 priority pollutants in wastewater(4). These procedures include (a) 12 methods for gas-liquid and high performance liquid chromatography with specific detectors and (b) 3 methods using gas chromatography/mass spectrometry for multiple measurements. Because of the high complexity of the organic contaminants in wastewaters, GC/MS methods are usually the methods of choice in most analyses. For example, Method 625 is a general screening method for extractable priority pollutants. This method, while very useful for most applications, has several shortcomings such as: 1) the initial base/neutral extraction may cause significant reduced recoveries of phenol, 2-methyl phenol and 2,4-dimethylphenol; 2) the bases such as benzidines and other amines are difficult to chromatograph; 3) presence of fatty acids in wastewater usually results in very complex chromatograms; 4) two extractable fractions are obtained using this method, which

usually require two GC/MS analyses.

Another approach was funded by the USEPA to modify and develop an analytical approach to enable the analysis of raw wastewater and sludge samples. Results of this study were detailed in a report submitted to the EPA by F.P. DeWalle and E.S.K. Chian(5). The analytical method used in this study involved an acid/neutral extraction followed by a base extraction. Due to the high concentrations of organic interferences in this type of samples, special separation and cleanup procedures such as gel permeation chromatography, florisil chromatography and cesium silicate chromatography were required to facilitate analysis for individual group of compounds. This method produced a base fraction, 3 neutral fractions and 1 acid fraction which was derivatized with diazomethane.

The results of this study indicate detection limits of about 1 ug/L for the extractable organics and 5-10 ug/L in sludge samples. Although this method offers cleaner fractions for processing, there are several drawbacks that discourage its use for routine monitoring. Firstly, the procedure is time consuming and labor intensive, and consequently reduces sample throughput. Secondly, the multi-fraction approach results in more losses of organics due to the separation processes. Thirdly, the cost of analysis will be very high as it involves a minimum of three extractable fractions and one volatile fraction.

Since MOE has proposed a regulatory and compliance program which will require municipal STP's to begin monitoring for the presence of hazardous contaminants in their raw sewage and final

effluent, cost-effective analytical methodology is needed. The objective of this study is therefore to develop a reliable protocol which will maximize sample throughput and provides the required accuracy, sensitivity and precision for such analysis. To achieve the project objectives, two novel approaches have been investigated in this study : a) simultaneous in-situ acetylation and extraction coupled with the purge and trap method and b) dynamic thermal stripping method. Although the latter method showed promising results for most of the compounds under investigation, the simultaneous extraction and acetylation method was the method of choice due to the ease of automation.

The Ontario Ministry of the Environment has preliminary identified more than 84 compounds for this study. Based on the frequency of occurrence and concentration levels of organics reported in the previous studies (1-3), a target list was generated to represent a broad spectrum of organics and used throughout the study to test the protocol. These target compounds are listed in Table 1.

TABLE 1. LIST OF TARGET ORGANICS

Halogenated Aliphatics

Chloroform
Dichlorobromomethane
Tetrachloroethylene
Trichloroethylene
1,1,1-Trichloroethane
Vinyl Chloride

Halogenated Aromatics

Chlorobenzene
Hexachlorobenzene
1,4-Dichlorobenzene
Aroclor 1254

Non-Halogenated Aromatics

Benzene
Benzo(a)pyrene
Chrysene
Dibenzo(a,h)anthracene
Naphthalene
Styrene

Miscellaneous

Dimethyl Disulphide
1,4-Dioxane

Pesticides

Dieldrin
gamma-BHC
Diazinon
p,p'-DDT
Chlordane
Atrazine

Phenols

Phenol
Pentachlorophenol
o-Cresol
2,4,6-Trichlorophenol

Phthalate Esters

Bis(ethylhexyl)phthalate

Amines

Aniline

Ethers

Diethyl Ether

OVERALL APPROACH

The finalized approach used for the determination of the target organic pollutants in the reagent water, raw sewage and final effluent involved the addition of methylene chloride, potassium bicarbonate and acetic anhydride to the water sample. The sample was then extracted for 15 minutes and the the organic phase collected. Under the basic pH, the neutral and basic compounds were extracted into the methylene chloride layer. The phenolic compounds were converted by acetic anhydride to their respective acetates and the amines to their corresponding amides. These derivatized products were then extracted into the methylene chloride layer. Since the acids themselves were not derivatized by the acetic anhydride, they remained in the aqueous layer.

Using this method, all bases, neutrals and phenols can be extracted in one fraction , the overall recoveries of phenolics will greatly be increased because of the single extraction. Quantitation of the amines is better due to better peak shape. Since free fatty acids are known to be a major class of interferences in wastewater, using this method, they will stay in the aqueous layer, hence producing a cleaner extract for chromatographic analysis.

The compounds of concern were analysed without further cleanup by GC/MS using high resolution capillary column and selected ion monitoring mode. Quantition of the derivatized compounds were achieved using the labelled standards spiked into

the sample and analyzed under the same conditions. The rest of the underivatized compounds were based on the internal standard quantitation. Relative response factors obtained from the standard solutions were used as correction factors in the calculation of the organic concentrations. This analytical method provides high sensitivity, high resolution of target organics from the background interferences and high selectivity of the detector responses.

The volatile organic fraction was analysed using the purge and trap method by GC/MS using capillary column in the full scan mode. A 30 ml aliquot of the water sample is used for analysis.

A total of two fractions, a volatile and a semivolatile fraction, were obtained from each sample using this protocol. This approach eliminates the cleanup steps for the semivolatile fraction, improves the recovery of the phenolic compounds, provides better detection of the amines and substantially increase sample throughput.

EXPERIMENTAL

SIMULTANEOUS IN-SITU ACETYLATION AND EXTRACTION

All raw sewage and final effluent samples were collected from Main Treatment Plant in Toronto and provided by the Water Resources Branch of the Ministry of the Environment. A 500 ml sample of wastewater was accurately measured and fortified with several labelled compounds to monitor the extraction efficiency.

A 50 ml aliquot of methylene chloride and a surrogate mixture of phenols were added to the water sample in an erlenmeyer flask and mixed thoroughly for 10 minutes on a magnetic stirrer. Approximate 10g of anhydrous KHCO_3 was then added and stirred for another 5 minutes to ensure complete dissolution of the solid. An accurately measured 2.5 ml of acetic anhydride was finally added to the flask and vigorously stirred for 15 minutes. At the end of the extraction, the two phases were allowed to separate in a separatory funnel and the organic layer collected in a round bottom flask. When necessary, the samples were subjected to centrifugation to break the emulsions. The extractive acetylation was repeated twice with 50 ml of CH_2Cl_2 and 15 minutes of stirring each time. The methylene chloride layers were then combined, passed through pre-washed sodium sulfate, and concentrated to about 2-3 ml. The extract was further concentrated to 1 ml with a gentle stream of nitrogen and spiked with an internal standard prior to autosampler processing.

INSTRUMENTATION

Analysis of the extractable organics was performed on a Hewlett Packard Mass Selective Detector system interfaced with a HP 5890 gas chromatograph. Chromatographic separation was achieved with a J & W DB-5 fused silica capillary column (30 m x 0.25mm, 0.25um film thickness). The chromatographic conditions were as follows: Injector temperature, 250°C; temperature program, 70°C hold for 1 min, 20°C/min to 170°C, 5°C/min to 200°C, 20°C/min to 280°C with a final hold of 12 minutes. The interface temperature was 285°C. The linear velocity of the helium carrier gas was 30 cm/sec. Samples were introduced in the splitless mode with the solvent vent closed for 0.5 min after injection.

Mass detector conditions were as follows: electron energy, 70eV; MS operating mode, SIM; Dwell time per ion, 50 msec. Electron impact spectral data was acquired and processed by the HP 9816 computer and the corresponding MSD software.

Volatile organics and the sorbent tube samples were analysed on a UNACON 810B automatic concentrator (Envirochem Inc.). This instrument was equipped with a sparging assembly for analysis of volatile organics in water, and a desorber unit for thermal desorption of the sorbent tubes. The internal trapping system consisted of a large bore primary trap which permitted preliminary separation of compounds of interest from the carrier gas. The secondary small bore trap was approximately one-tenth the capacity of the first trap and allowed for an efficient transfer of the retained compounds to the capillary column.

Initial concentrator operating conditions were: transfer

line to MS, 280°C; valve compartment, 250°C; trap 1 desorbed at 300°C with a helium flow of 50ml/min for 5 minutes; trap 2 desorbed at 300°C with a helium flow of 3 ml/min for 5 minutes onto a 30 m DB-5 column.

For volatile organics, a 30 ml aliquot of the water sample was transferred to a 50 ml sparging vial and purged for 15 minutes at a flow rate of 50 ml/min at 50°C. For sorbent tube analysis, the tube was placed inside a desorber unit with both ends tightly sealed by vespel/graphite ferrules. The sorbent tube was then rapidly heated to 300°C with a helium flow of 50 ml/min for 15 minutes to release the organics into the first trap. By sequential heating of the first and second trap, a discrete narrow-band plug of sample was deposited on the head of the column.

RESULTS AND DISCUSSION

EXTRACTION METHOD

Recoveries of target organics using the in-situ acetylation and extraction technique have been conducted in final effluent, raw sewage and reagent water matrices. Results of these analyses are presented in Tables I to III.

Initial extraction using 500 ml of organic free water indicates good recoveries for phenolic compounds which range from 80% for pentachlorophenol to 94% for o-cresol. These recoveries are far better than those of EPA method 625 due to the fact that the phenols were extracted and derivatized in the same

TABLE I

RECOVERIES OF THE TARGET ORGANICS IN REAGENT WATER

PARAMETER	SPIKED LEVEL (ng)	MEAN LEVEL DETECTED (ng)	% CV	MEAN % REC
Phenol(A)	10.26	8.82	10.7	86.0
Aniline(A)	15.00	14.06	9.7	93.7
O-Cresol(A)	10.58	12.09	8.8	94.9
Napthalene	10.18	9.31	15.4	91.5
2,4,6 TCP(A)	9.94	9.12	5.5	91.7
HCB	10.10	9.03	5.4	89.4
Atrazine	11.26	10.76	2.5	95.6
Gamma-BHC	17.00	17.80	2.9	104.7
Diazinon	21.50	19.31	4.4	89.8
Pentachlorophenol(A)	9.94	7.93	9.7	79.8
Alpha-Chlordane	12.70	11.82	8.0	93.1
Dieldrin	10.12	9.37	5.4	92.6
4,4'-DDT	10.06	9.37	3.8	96.7
Chrysene	22.50	21.94	8.5	97.5
Bis(Ethyl-Hexyl)Phthalate	10.32	7.63	6.0	74.0
Benzo(A)Pyrene	10.20	8.98	7.1	88.0
Dibenzo(A,H)Anthracene	21.20	19.52	6.1	92.1

(A) = Acetylated

TABLE II

RECOVERIES OF THE TARGET ORGANICS IN FINAL EFFLUENT

PARAMETER	SPIKED LEVEL (ng)	MEAN LEVEL DETECTED (ng)	% CV	MEAN % REC
Phenol(A)	10.26	7.75	7.2	75.5
Aniline(A)	15.00	14.42	13.5	96.1
O-Cresol(A)	10.58	11.07	24.5	104.6
Napthalene	10.18	9.03	7.8	91.0
2,4,6 TCP(A)	9.94	8.47	4.4	91.0
HCB	10.10	9.60	3.7	95.0
Atrazine	11.26	9.20	9.8	81.7
Gamma-BHC	17.00	16.28	5.2	95.8
Diazinon	21.50	18.14	7.2	84.4
Pentachlorophenol(A)	9.94	8.58	4.3	88.3
Alpha-Chlordane	12.70	9.16	4.5	72.1
Dieldrin	10.12	8.69	3.5	85.8
4,4'-DDT	10.06	11.43	16.17	94.4
Chrysene	22.50	16.41	2.2	72.9
Bis(Ethyl-Hexyl)Phthalate	10.32	7.76	12.9	75.7
Benzo(A)Pyrene	10.20	9.43	6.5	92.5
Dibenzo(A,H)Anthracene	21.20	20.41	7.5	96.3

(A) = Acetylated

TABLE III

RECOVERIES OF THE TARGET ORGANICS IN RAW SEWAGE

PARAMETER	SPIKED LEVEL (ng)	MEAN LEVEL DETECTED (ng)	% CV	MEAN % REC
Phenol(A)	10.26	7.52	6.0	73.3
Aniline(A)	15.00	13.39	6.1	89.3
O-Cresol(A)	10.58	7.66	9.6	72.4
Napthalene	10.18	8.92	6.5	87.6
2,4,6 TCP(A)	9.94	9.09	2.7	91.4
HCB	10.10	10.80	2.8	106.9
Atrazine	11.26	11.18	17.1	99.3
Gamma-BHC	17.00	13.85	9.1	81.5
Diazinon	21.50	22.28	3.6	103.6
Pentachlorophenol(A)	9.94	8.73	9.8	87.8
Alpha-Chlordane	12.70	10.20	9.9	80.3
Dieldrin	10.12	6.92	6.0	68.4
4,4'-DDT	10.06	7.98	7.9	79.3
Chrysene	22.50	16.86	7.0	74.9
Bis(Ethyl-Hexyl)Phthalate	10.32	10.68	14.2	103.5
Benzo(A)Pyrene	10.20	10.31	11.3	101.0
Dibenzo(A,H)Anthracene	21.20	19.30	4.6	91.0

(A) = Acetylated

fraction, thus eliminating losses in the initial base/neutral fraction. In addition to the phenols, amines such as aniline were also converted to amides which greatly improved the chromatography and ultimately the sensitivity.

The recoveries of the phenols were calculated based on those obtained from the repective standards under the same conditions as detailed in the experimental section. The quality of the data will not be affected since both standards and samples were treated the same.

It is well known in the EPA method 625 that some organo-chlorine pesticides such as alpha and beta BHC, endosulfan I and II and endrin are subject to decomposition under the alkaline conditions of the extraction step. By using this in-situ acetylation method in which a weaker base such as potassium bicarbonate was used, the recoveries and reproducibility of these compounds were found to improve significantly.

Experiments were also carried out to determine the recoveries of the EPA priority pollutants using this procedure. Results indicate that the benzidine and 3,3'-dichlorobenzidine were acetylated. Good recoveries of most of the EPA compounds including N-nitroso-di-n-propylamine, N-nitroso-dihenylamine, diphenyl hydrazine and isophorone. Among all the EPA compounds, 4,6 dinitrophenol and 2,4 dinitrophenol were found to have low recoveries. Further work will be conducted to investigate this problem.

The isolation and identification of trace hazardous organics from complex background is the prime aim of any wastewater

extraction procedure. In most methods used today, gel permeation chromatography or other column cleanup procedures are frequently employed to reduce interferences, thus resulting longer sample processing time. Using this in-situ acetylation method, the fatty acids which are present at high concentrations in municipal wastewater would not be derivatized and hence remain in the aqueous layer. This greatly reduces the chromatographic background and eliminates the need for a cleanup step. On the other hand, by eliminating the acid compounds, the phenoxy herbicides which contain the carboxylic acid functional group would not be detected.

Using this approach, all bases, neutrals and phenols will be extracted in the same fraction. Hence, only one extractable and one volatile fraction are needed for each sample. With autosamplers for sample introduction, coupled with extensive software development for data processing, a high sample throughput with minimum manpower requirement can be achieved.

VOLATILE ORGANIC ANALYSIS

In combination with the in-situ acetylation method, a capillary purge and trap method was used for the analysis of the volatile organics. Depending on the sample volume, this technique can be used to produce sub-ppb detection limits for a large number of volatile compounds.

Table IV Mean % Recoveries of Volatile Organics Using Purge and Trap Method

Compound	Mean Recovery (%)	C.V. (%)
Vinyl Chloride	93.9	10.3
Chloroform	81.2	3.6
1,1,1-Trichloroethane	102.5	9.5
Benzene	91.4	7.2
Bromodichloroethane	96.0	4.9
Trichloroethylene	91.1	7.4
Tetrachloroethylene	80.3	7.6
Chlorobenzene	78.5	5.4
Diethyl Ether	66.4	9.7
Dimethyl Disulphide	80.0	7.9
Styrene	91.1	9.2
1,4-Dioxane	0.3	8.5

The purging efficiency and reproducibility of the target compounds are depicted in Table IV. The purging efficiency is a reflection of the percentage of the amount of a specific compound that can be recovered from water. In these analyses, the accuracy of the results was not affected by the recoveries since samples and standards were analysed using the same procedures.

In this study, it was observed that the recovery of 1,4-dioxane was extremely poor. This was mainly due to the miscibility of this compound with water. With an increase in temperature during sparging and a higher ratio of gas flow versus sample size, a better recovery was obtained. Recoveries of the other volatile compounds were very good. They ranged from 66% for diethyl ether to 102% for 1,1,1-Trichloroethane. The coefficients

of variation for majority of the compounds were well within 10%.

DETECTION SYSTEM

In this project, a Hewlett Packard mass selective detector was used. Customized software was developed to allow unattended sample handling and data processing.

This detector was exclusively used in the "Selected Ion Monitoring" mode to obtain the best possible sensitivity, improved quantitation and increase computer storage capability. The sensitivity of the SIM mode (10-100pg) was sufficient to detect concentrations of organics found in raw sewage or final effluent. The readily detectable limits of 100 pg for most of the hazardous organics enabled only 500 ml of sample to be routinely processed.

For each parameter in the target list, three ions were selected based on their intensity and uniqueness. To confirm the identity, retention times and the ratio of the two qualifying ions to the quantitation ion had to be within the strictest tolerance. Two time reference peaks were chosen to help define the relative time window for each parameter. Internal standard quantitation method was used in these analyses.

For Aroclor 1254, a quantitation ion was selected from each of trichloro, tetrachloro, pentachloro and hexachloro cogener groups, these, coupled with the two qualifying ions and scanning over the entire range of the expected retention times, provided positive confirmation of the presence of Aroclor 1254. Quantitation was achieved by the summation of these target peaks.

SOFTWARE APPROACH

Automation is becoming an important aspect in most analytical chemistry laboratories. There are obvious reasons for increasing automation such as : less time is spent in sample introduction and repetitive laboratory endeavour; greater standardization of data collection and reduction operations results; more attention is paid to problem solving; and flexibility is increased.

In order to assure the accuracy of the detection and maximum efficiency for sample processing and interpretation, extensive software development was required. Our major objective in this regard was to effectively identify and quantify the compounds of interest under various adverse chromatographic conditions such as background interference, overloading and saturation problems which commonly occur in this type of analysis.

Software development from these concerns resulted in the creation of a series of independent programs. These programs were developed to ease the process of data reduction and achieve a high level of automaticity. Furthermore, they can either be linked together or processed individually. Typical examples of some of the programs are:

- * a program to extract and integrate ions of interest and to display the information in a way which is easily witnessed by the operator (Figure 3)
- * a program to detect the overloading and saturated peaks
- * a program to provide spectra of each peak in the chromatgram

- * a program to automatically initiate and set up an autosequencing method
- * the "Mann Link", a top view type program that adds a new dimension to autosequencing capability and flexibility
- * a full and sophisticated background subtracted spectra library search program
- * a program that allows a visual pattern recognition format for Aroclor 1254 (Figure 6)
- * a program to conduct daily QA/QC and compare previous results automatically

Following each acquisition, data reduction macros were used to display the three ions for each compound, and to monitor the quality of the integration. Separate tests were automatically run to flag any saturated or overloading peaks. Subsequent internal standard reports were processed and automatically printed.

Peak identification in the report program had to meet the following criteria:

- i) the strict adherence to the correct relative retention time window with respect to the two time reference peaks
- ii) the presence of the designated quantitation ion and two qualifying ions
- iii) the correct ion ratios of the two qualifiers to the quantitation ion
- iv) the peak area of the internal standard must be within a specific tolerance

Following these acquisitions and reports, further data reduction macros were automatically invoked to display ions and

qualifiers for Aroclor 1254. Special programming displayed Aroclor 1254 in such a way as to allow visual pattern recognition by the operator similar to that being done with the electron capture detector. Several options then followed: library search for a number of peaks or every peak in the chromatogram; display spectra of every peak; continue injection of the same batch of samples or process another batch using different macros; transfer the data to another media and delete the file on hard disk.

In every aspect these types of software development are to allow complete flexibility and to achieve maximum sample throughput .

ACKNOWLEDGEMENTS

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APPLICATION OF GAS CHROMATOGRAPHY
WITH HELIUM MICROWAVE PLASMA DETECTION
TO THE RAPID SCREENING OF CHEMICAL WASTES

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Introduction

The helium microwave plasma, particularly at atmospheric pressure using a Beenakker TM₀₁₀ cavity, shows a great deal of promise as a gas chromatographic detector because of its ability to detect a very wide range of elements, both metal and non-metal^{1,2}. By incorporating a polychromator as a part of the instrument, one can derive the benefits of simultaneous, multi-element detection such as higher speed of analysis and smaller sample requirements. This paper describes a helium microwave plasma detector for GC that was developed by the authors.

Experimental

Figure 1 shows a block diagram of the entire instrument. Helium, after being purified, goes into the GC and the gas control unit. This provides for solvent venting as well as supplying helium plasma support flow. Independent of the column flow, scavenging or doping gases can also be introduced through this unit. A scavenger is a gas such as nitrogen or oxygen that when introduced into the plasma at low concentrations removes carbon deposits on the torch walls. There is also the capability of introducing a doping gas. Some workers² have seen improved sensitivities for a number of elements by doping the plasma with a small flow of hydrogen. The GC-plasma interface incorporates fluidic logic switching. The solvent is vented so that it does not disrupt the plasma. Subsequent GC eluent reaches the plasma without having to pass through valves. A 200 watt microwave generator is linked to the Beenakker cavity. A nine channel polychromator was built

to provide simultaneous detection. It has chart recorder outputs and also a socket for direct connection to a Spectra-Physics SP 4000 computer. The interface oven and Beenakker cavity are rigidly mounted together on a common support. This support is on an X, Y, Z stage which allows precise and reproducible positioning of the Beenakker cavity in front of the entrance optics to the spectrometer. The interface oven consists essentially of a heated aluminum block containing a manifold which has two side arms.

Figure 2 shows the optical layout of the multi-channel spectrometer. Light from the plasma is focused by a lens onto the dual entrance slits. The two entrance slits allow square wave wavelength modulation for background correction. A chopper wheel lets light alternately through one slit or the other. On the far side of the Rowland circle is a concave holographic grating having 1180 lines per millimetre. The light is dispersed by the grating across the Rowland circle to the nine exit slits located on the bridge on the far side.

Behind each slit is a mirror at 45° which reflects the light up to the end-on photomultiplier tube for that element located on top of the bridge. There is an order sorting filter in front of each PMT.

The multichannel spectrometer was aligned to look at the following nine elements: carbon, phosphorus, mercury, bromine, chlorine, sulfur, fluorine, nitrogen, and oxygen. Background correction for each analytical line was accomplished by monitoring the intensity at the peak wavelength and subtracting

from it the intensity 1.0 nm away from the peak. A synchronous detector circuit was incorporated into the electronics of each channel to provide this function.

One of the problems many researchers have seen is the very large background levels of nitrogen and oxygen. This was observed in this work as well. Oxygen exists as an impurity in the helium gas supply, it can come from column bleed, it can come from etching of the quartz torch and it can also enter the plasma by back diffusion from the open end of the torch. Nitrogen can arise from the first and fourth of these sources as well.

Because it was thought that back diffusion from the open end of the torch might be a significant source of nitrogen and oxygen, a flush cell was devised to be attached to the end of the discharge tube. One end of the flush cell was connected to the discharge tube with a straight Swage union. Additional helium was forced in through a sidearm. All of the helium exhausts through another sidearm and through a length of aluminum tubing. It was hoped that the length of the path for back diffusion was great enough to minimize back diffusion significantly. The levels of oxygen and nitrogen were seen to go down when the flush cell was used. Unfortunately, the reductions were not enough to allow reliable detection of nitrogen-and oxygen-bearing compounds in chromatographic eluents.

Results

Experiments were carried out to determine the dynamic range of each channel. For these experiments, the response of each

signal channel was integrated to compensate for changes in peak shape as a function of the amount of material eluting in the column eluent. Various concentrations of the test compounds were used, up to a maximum of 5%. Calibration curves were constructed by plotting the response as integrated area versus amount of each element injected on logarithmic scales. A typical calibration curve is the one for sulfur using dibutylsulfide, shown in Figure 3.

In general, the linear dynamic range of each channel extends up to approximately 50 ng of each element. Under the conditions used for these determinations, the peak width varied between 2 and 3 seconds for each compound. Since the detection limits for the elements in general are between 10 and 20 pg/sec, the absolute detection limit for most of the elements is approximately 50 pg. Thus the linear dynamic range for the elements determined is approximately 1,000.

One of the most important things to determine was the position of maximum intensity for the different elements within the plasma torch. A part of this was the determination of how the position of maximum intensity changed as a function of helium flow rate and applied power. Profiling studies of element emission intensity across the torch were done by moving the Beenakker cavity with respect to the spectrometer. Figure 4 shows the carbon emission intensity across the torch with the helium flow ranging from 42 to 210 mL/min. The maximum intensity was in the centre of the torch and changing the helium flow did not change the position of the maximum intensity. This was observed for all of the elements studied.

Profiles were also done across the torch while varying the applied power. Figure 5 shows such a profile for phosphorus. Again, the position of maximum intensity is at the centre and it does not vary with incident power. The general conclusion that can be drawn from the profiling studies was that all the elements had their maximum intensity at the centre of the torch and that varying power or helium flow did not change the position of maximum intensity. This was an important result because it meant that when these parameters are varied it is not necessary to reposition the cavity with respect the spectrometer for maximum sensitivity.

Figure 6 shows the intensity of the chlorine signal as a function of incident power. Increasing the power increases the signal intensity and this is typical of the elements that were studied.

The signal intensity was also monitored while varying the helium flow rate. Figure 7 shows the results for carbon. Again, this is typical of the other elements. Reducing the helium flow would increase the emission intensity, probably, because at a lower helium flow the analyte has a longer residence time in the plasma.

Based on the studies of emission intensity as a function of power and helium flow, a set of optimum conditions were established that would be the best overall for all of the elements. These compromise conditions are 85 watts of incident power and 50 mL/min of helium plasma flow. Using these conditions detection limits and selectivities against carbon were determined. These are shown in Table I.

Conclusions

The helium microwave plasma has demonstrated many desirable characteristics as a simultaneous multielement detector for gas chromatography. It has also been widely studied as a detector in non-chromatographic instrumentation. The plasma has been used to quantify such species as sulfur gases³, carbonates in coal fly ash⁴, heavy metal salts⁵, tetraalkylleads⁶, and volatile organo-mercury compounds⁷.

Acknowledgement

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TABLE 1

Detection Limits and Selectivities
for Simultaneous Determination a)

<u>Element</u>	<u>Detection Limit,</u> <u>pg/s</u>	<u>Selectivity vs. C</u> b)
Br	27	180
C	4	1
Cl	12	200
F	11	366
Hg	1	131
P	4	1370
S	32	60

a) plasma operating conditions 85 watts incident power, 0-2 watts reflected power, 50 mL/min He plasma support flow rate.

b) using n-decane

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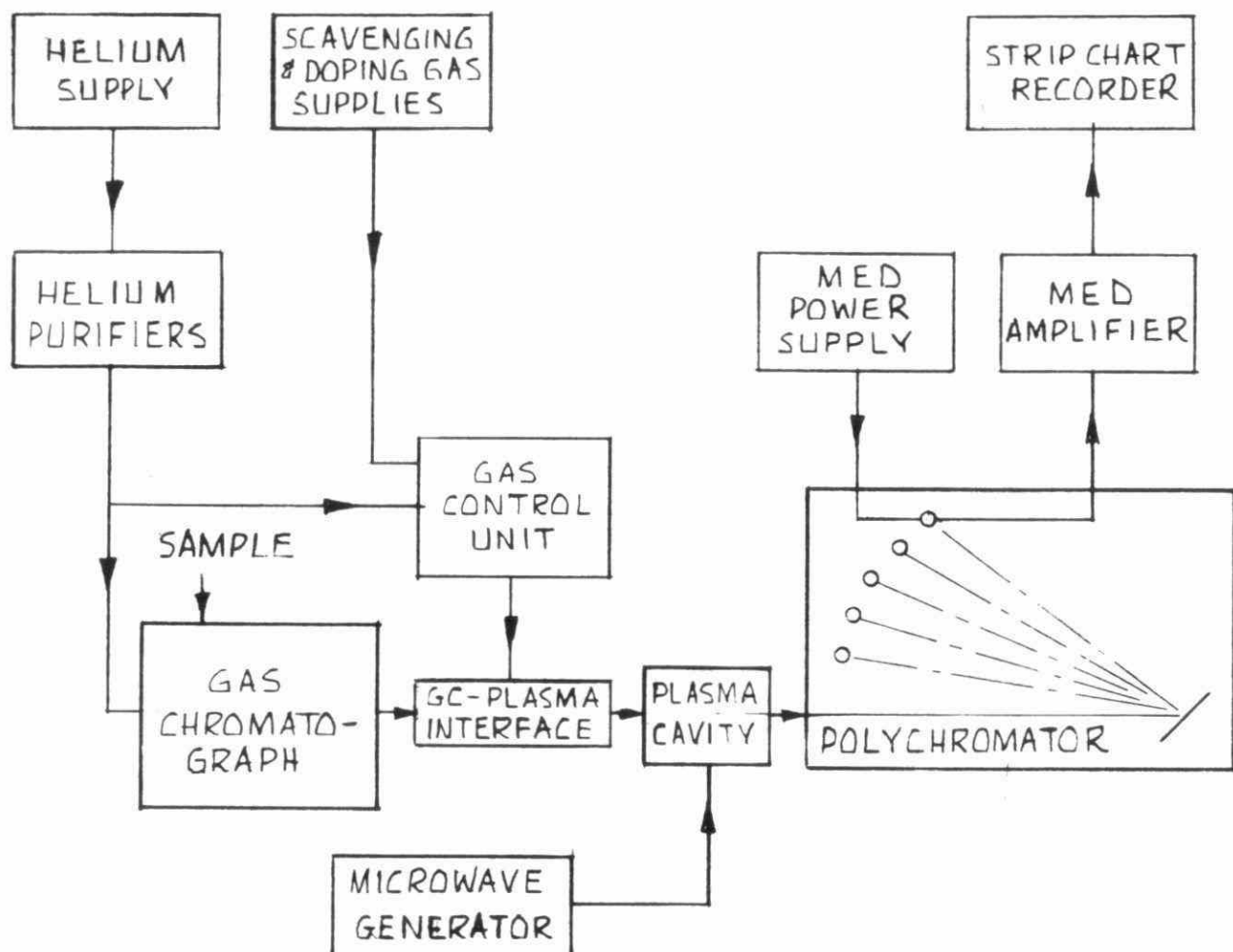


FIG. 1

BLOCK DIAGRAM OF THE MED 650

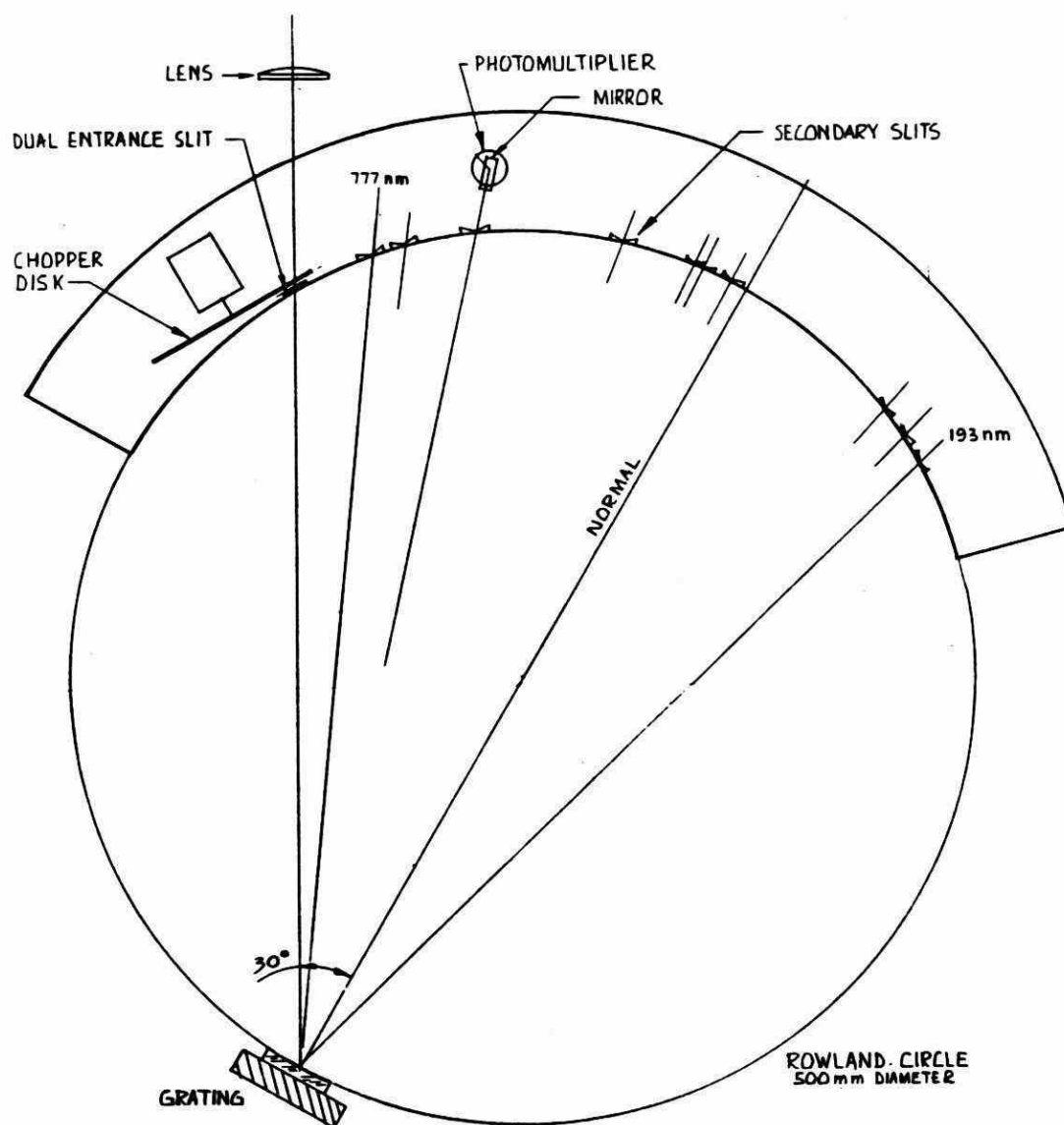


FIG. 2

OPTICAL LAYOUT OF MULTICHANNEL SPECTROMETER

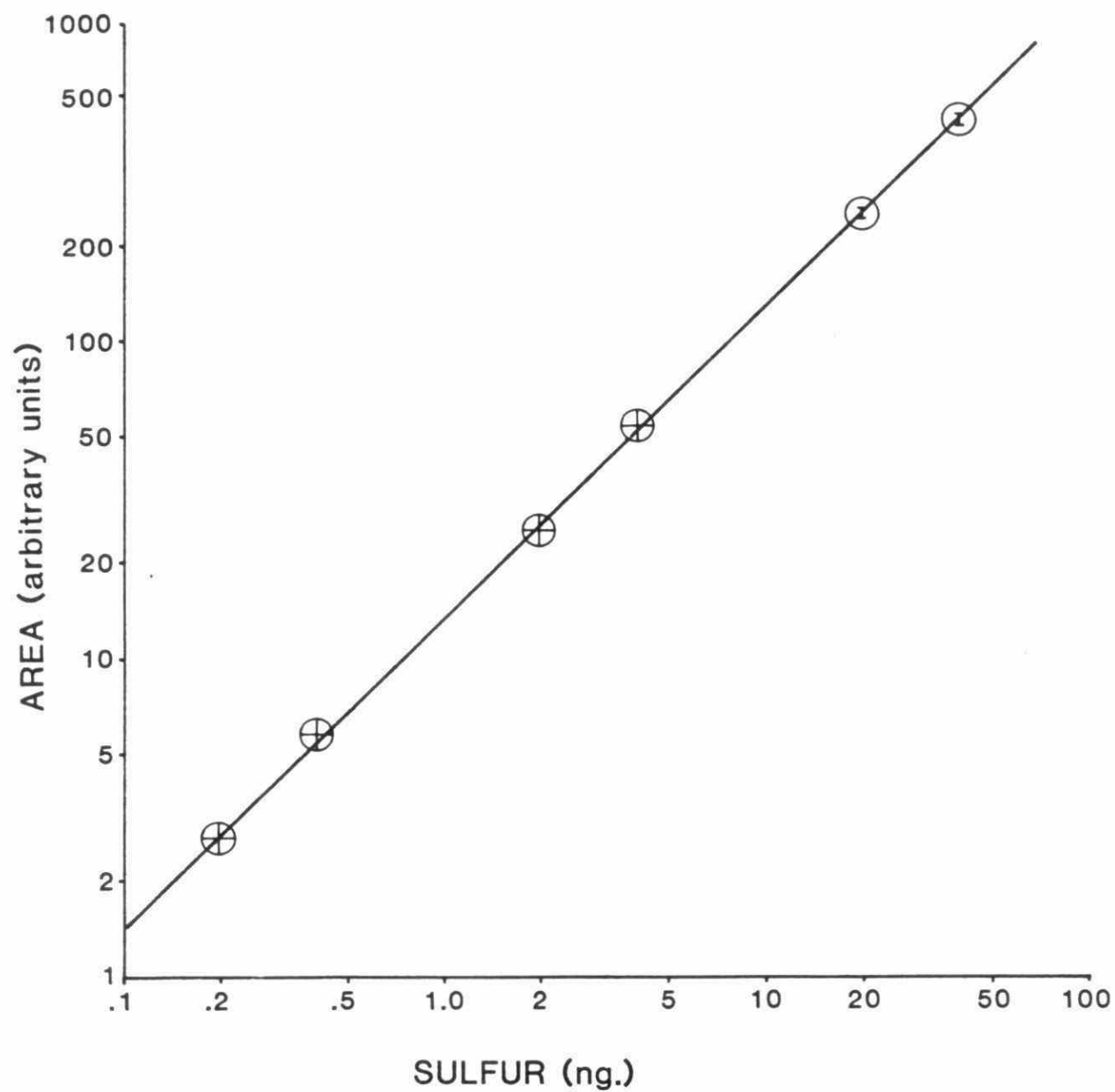


FIG. 3: Calibration Curve for Sulfur Using Dibutylsulfide

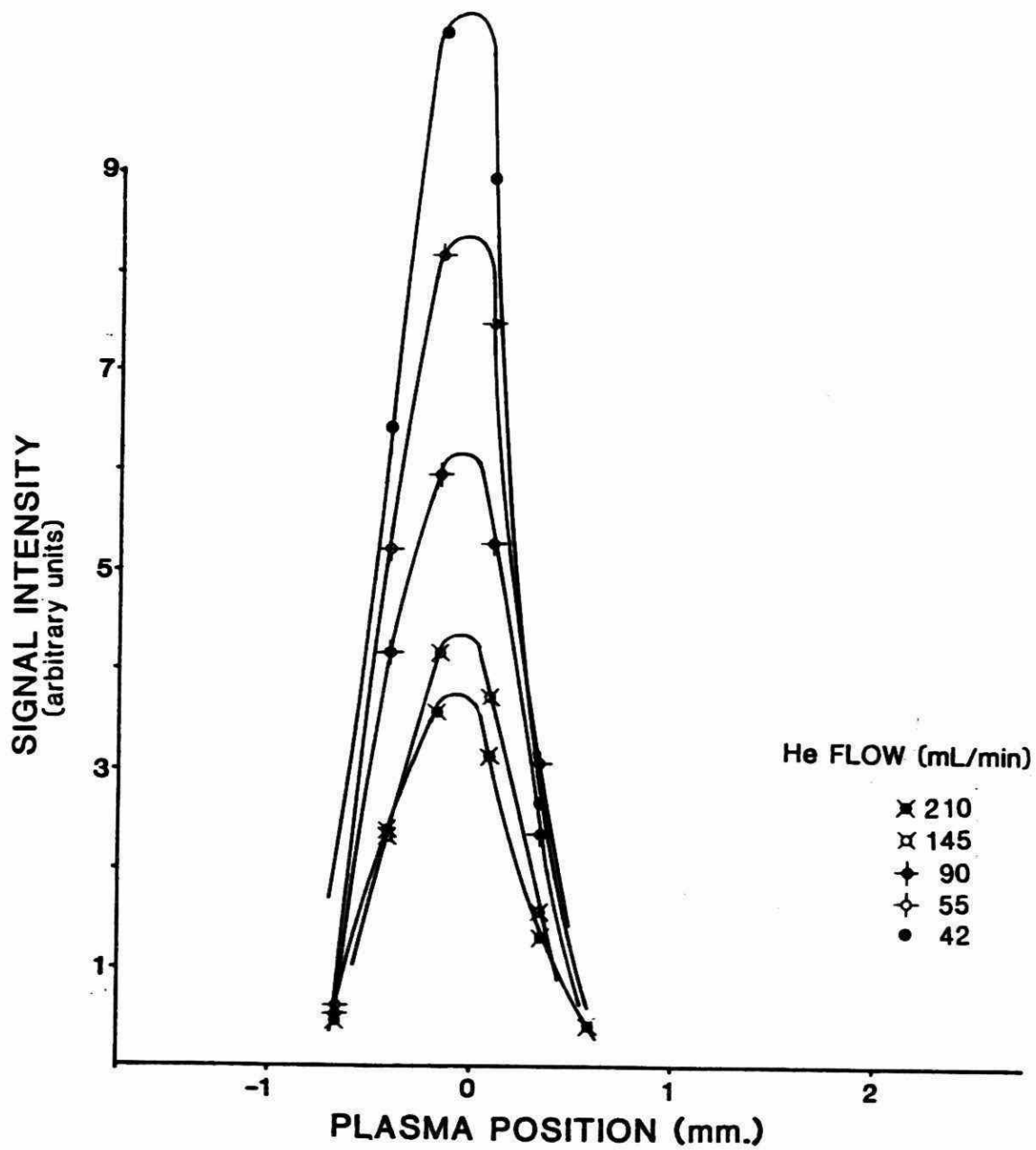


FIG. 4: Carbon Profile at Different Flows (85 Watts)

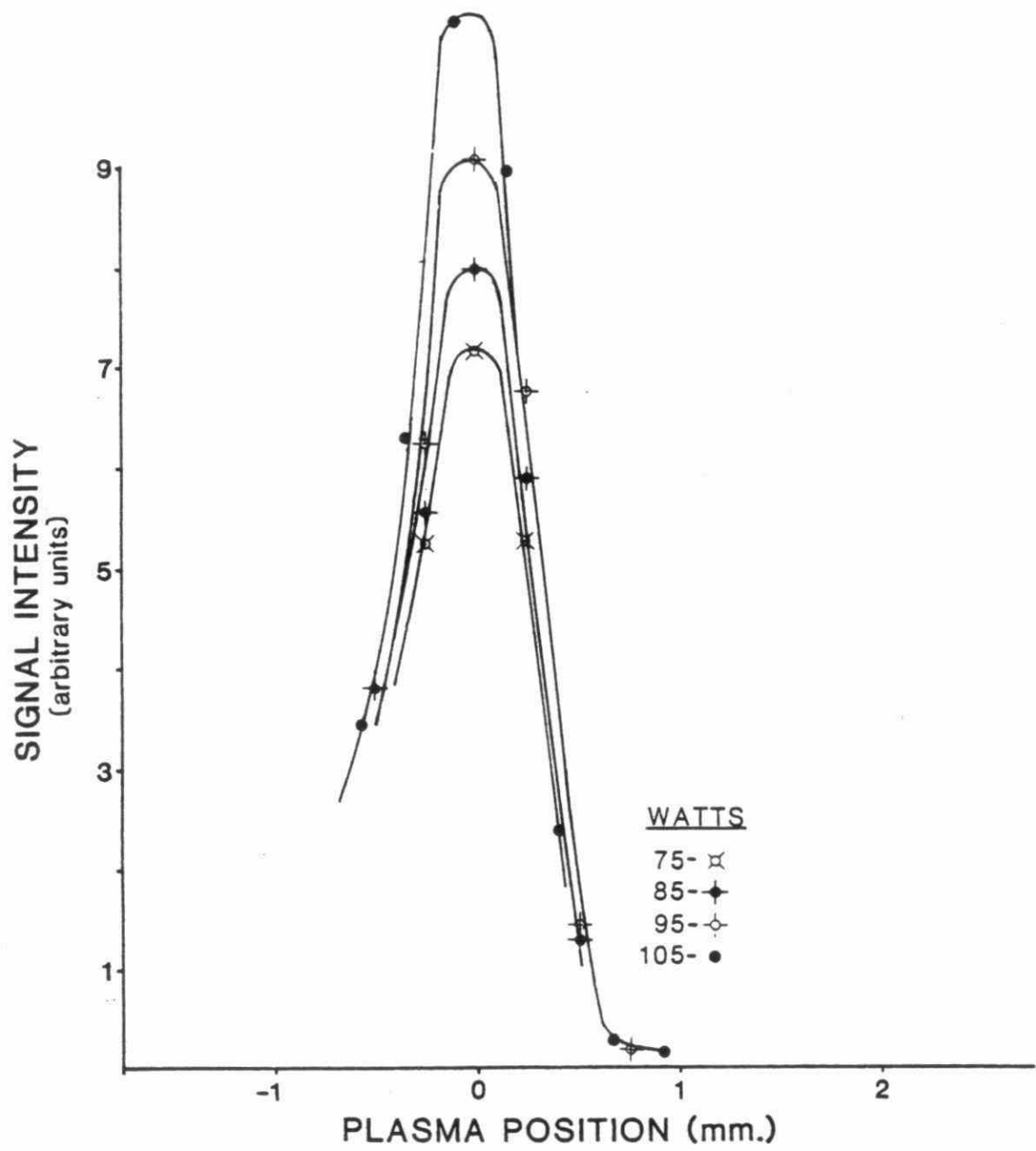


FIG. 5: Phosphorous Profile at Different Powers

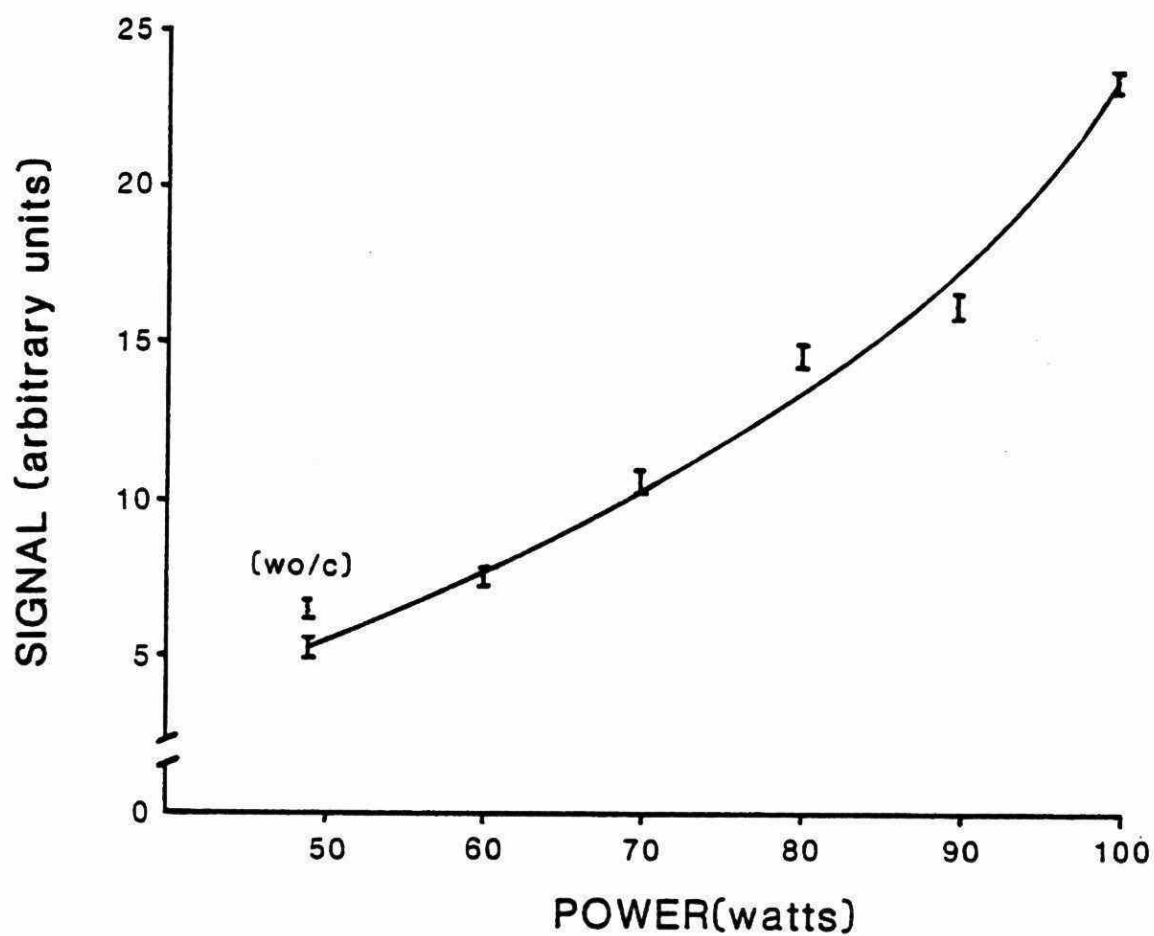


Figure 6

Chlorine Signal versus Incident Power; He plasma flow rate = 42 mL/min

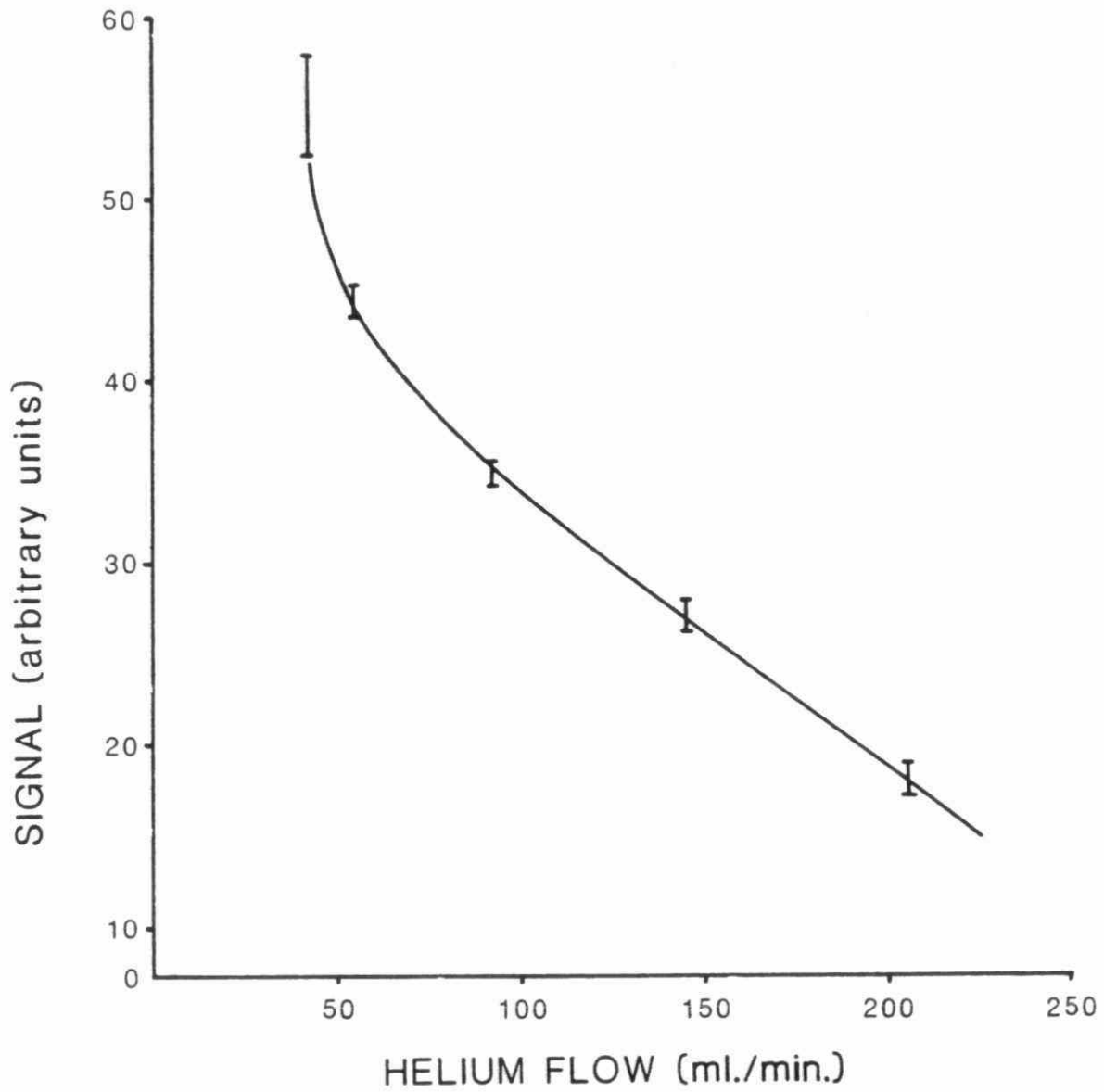


FIG. 7: Carbon Signal Versus Helium Flow
(incident power = 75 Watts)

Technology Transfer Conference
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The Sheraton Centre, Toronto, Ontario

Presentation:

"Development of Methods Suitable for Use in the
Ames Salmonella Mutagenicity Assay for the
Collection of Organics Present in Groundwater
and Landfill Site Matrix Materials"

by: N. Belson, A. J. Horton, K. Shaw and G. H. Thomas
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INTRODUCTION

There is a need for the development and validation of test protocols to assess the degree of leachability and mobility of potentially hazardous materials contained in landfill disposal sites. A detailed chemical analysis and identification of all chemical species in leachates and groundwater is desirable, but such survey type analyses are time consuming and costly. In addition, because of synergistic, antagonistic or other chemical and/or microbiological transformations which may take place in the environment, chemical analysis alone may not provide adequate assessment of the potential health risks involved.

Short-term biological tests are attracting interest as a basis for possible regulatory purposes. The reason is that these tests provide some predictive value regarding health effects. The overall goal of this program is to develop and validate concentration methods for organic chemicals from groundwater and solid matrix material for use with the Ames bioassay.

LITERATURE REVIEW

The initial phase of the study was a literature search undertaken to collect existing data regarding organic chemicals present in leachates and in contaminated groundwater or surface water associated with sanitary landfill or hazardous waste disposal operations. This was accomplished in three stages:

- Computerized searches of key words in numerous data bases including the following: QL, Toxline, NTIS, Enviroline, Environmental Bibliography, Chemical Abstracts, Pollution Abstracts, Water Resources Abstracts and D.O.E. Energy.
- Key papers were ordered from the references listed in the computer search. The papers were numbered, abstracted, filed and listed. Additional papers were ordered from the references listed in the key papers. This leachate Master List is being continually updated as the program continues.
- From the list, papers were selected for detailed critical evaluation. This applies especially to those papers dealing with organic chemicals found in landfill leachate/groundwater extracts. Special emphasis was also given to papers relating to methods used for the isolation/concentration of organic compounds present at low concentrations in aqueous solutions. In addition, papers relating to analytical methods used to characterize the organic content of solid matrix material from landfill sites received special attention.

From this literature review, lists of organic compounds were compiled. The lists were assembled so that they served several purposes.

- It provides an alphabetical reference of the various compounds identified at problem sites.

- It defines the pollutant group into which the compound falls, viz:
 - 1) conventional pollutants, e.g. COD, TOC, TOX, phenols, etc.
 - 2) priority pollutants (consent decree)
 - 3) Section 311 compounds
 - 4) Resource Conservation & Recovery Act [RCRA]
(A - acute hazardous; H - hazardous; T - toxic)
- It classifies the compounds according to twelve (12) chemical classes similar to those used for priority pollutants.
 - 1) Alcohol
 - 2) Aliphatic
 - 3) Amine
 - 4) Aromatic - non-halogenated and halogenated aromatic compounds
 - 5) Ether
 - 6) Halocarbon-halogenated aliphatic compounds
 - 7) Miscellaneous - including selected priority pollutants, pH, BOD, TOC, COD, chloride, sulfate, phosphate, and other parameters generally used to characterize wastewaters.
 - 8) PCB
 - 9) Pesticide
 - 10) Phenol - including chloro- and nitro- phenols
 - 11) Phthalate
 - 12) Polynuclear Aromatic
- It specifies the ranges and concentrations encountered at actual hazardous waste disposal sites.
- It indicates frequency of occurrence at actual waste sites previously investigated.

In addition, the compounds listed were checked for positive mutagenicity/carcinogenicity activity. This was accomplished by comparison of the compounds listed with data present in appropriate mutagenicity references.

SELECTION OF CHEMICALS

Initially, it was necessary to select a limited number of chemical compounds for inclusion in an aqueous solution ("cocktail mix"). The purpose of this solution was to simulate, if possible, a landfill leachate in order to permit the validation of isolation/concentration techniques and measurement methods of the concentrates. The data acquired should provide information for predicting the behaviour of other related organic compounds when subjected to similar concentration techniques. The guidelines used for selecting the reference compounds were:

- to provide a wide selection of functional groups
- to represent, where possible, a range of pertinent physical properties such as volatility and polarity
- to choose compounds readily available in pure form at modest expense
- to avoid, where possible, multiple functional groups
- to choose, where possible, known water pollutants
- to select, when possible, halogenated derivatives of several parent compounds
- resistance to degradation
- to include known mutagenic compounds

The compounds selected for inclusion in the "cocktail mix" are listed in Table I. All the chemicals listed (with the exception of 2-Nitrofluorene) have appeared on lists relating to contaminants reported: viz. cancer risk assessment, chemical hazard information profiles (CHIP), and exposure assessments. The presumed mutagens on the list are indicated by means of an asterisk.

BIOLOGICAL PROPERTIES

Mutagenicity

One of the best known of the short-term bioassay systems is the bacterial test developed by Dr. Bruce Ames and co-workers in California. The assay uses mutant strains of the bacterium Salmonella typhimurium. Each strain contains a point mutation. This results in the mutant strain being unable to grow in the absence of histidine. In addition to the histidine mutation, each tester strain contains other modifications. One enables agents to permeate more easily into the bacteria. A second interferes with the bacteria's repair mechanism and permits the action of the mutagen to be expressed and detected by its assay.

Exposure of a tester strain to mutagenic substances results in some of the mutant cells reverting back to their wild state. In the process, the bacteria regain the ability to synthesize histidine and grow as colonies on histidine free media.

The frequency at which strains revert depends upon several factors, one of which is the potency of the agent tested. Mutagenic agents which are highly potent will induce a greater number of bacteria to revert than agents which produce less mutagenic damage. The addition of an activation complex is needed in some instances, as many substances which are non-mutagenic outside animal systems are structurally altered to a mutagenic form by metabolic processes within animal tissues. This function is accomplished in the Ames assay by the addition of a metabolic activation mixture (S-9).

Some investigators suggest bioassays are only useful when testing pure compounds because of problems associated with the interpretation of mutagenicity results. High concentrations of mildly toxic constituents or small quantities of highly toxic constituents in a complex mixture can suppress or prevent the detection of mutagenicity. The Ames bioassay has been used to assess the mutagenicity of complex mixtures, however, few studies have

been conducted to evaluate how mutagenic compounds behave in mixtures. The nature of the compounds selected for the cocktail mix and those known to exist in landfill leachates required that we investigate whether the proposed program of developing a biological monitor was feasible. This feasibility study was accomplished by evaluating how the selected compounds behaved within a combination of mixtures.

Toxicity

Toxicity (i.e. concentration that kills bacteria) tests are used to establish the maximum concentration of a compound that can be tested using the Ames assay. Mutagenicity and toxicity are sometimes closely related and it is necessary to test up to a toxic dose when screening for mutagenicity.

EXPERIMENTAL

All analytical work that was to be undertaken (both biological and chemical) was to be performed at chemical concentrations that were considered realistic in terms of the concentrations that might be expected in contaminated groundwater. The concentration chosen for validation studies was 1 µg/L (ppb) of the organic compounds in aqueous solution. It was expected that 50 L water samples would be used in the extraction/concentration procedures to be used. Before proceeding with any extraction/concentration techniques, it was necessary to assess the biological properties of the selected chemicals at the appropriate concentrations being considered to ensure that biological testing could be accomplished. If one assumed a 100% recovery, a concentrate containing 50 µg of each of the chemicals could be expected. Therefore, in order to assess the biological properties of the selected chemicals, concentrates in dimethyl sulfoxide (DMSO) of 50 µg/mL of each chemical was used.

Biological Properties of the Compounds Selected for the Cocktail Mix

a) Toxicity

Each of the twenty compounds was tested separately to determine the concentration that was toxic to Salmonella tester strain TA-100 in the absence of metabolic activation. All compounds were dissolved in dimethyl sulfoxide (DMSO) and the maximum amount of DMSO applied was 0.1 mL per plate, which is below the solvent toxicity. Toxicity tests were carried out using 0.1 mL of 2×10^8 suspension of tester strain TA-100. The standard plate incorporation assay was performed. Toxicity was assessed microscopically by examination of the background lawn and by comparison of the number of spontaneous revertant colonies. Most of the solid compounds were tested at half log doses up to 1000 $\mu\text{g}/\text{plate}$ unless prevented by solubility. The liquid compounds were assayed up to 0.5 mL (500 μL) per plate.

When all twenty compounds were mixed together at a concentration of 5 $\mu\text{g}/\text{plate}$ and less, there was evidence of slight toxicity. The slight toxicity did appear to have an effect on the mutagenicity of the cocktail mix. The toxicity did not prevent detecting the mixture as positive and mutagenic.

b) Mutagenicity

The four selected mutagens were tested using the Ames Salmonella mutagenicity plate incorporation assay procedure to determine dose-response data. The mutagens were tested for their known positive conditions (strains, concentration and activation requirements) up to the level of toxicity. Two Salmonella tester strains TA-98 and TA-100 were used in this investigation. The results are recorded in Table III.

Three of the four selected mutagens (2NF, BNA and B[a]P) showed positive dose-responses between 0.1 and 10 $\mu\text{g}/\text{plate}$. The fourth compound DBE was a weak, direct-acting mutagen with a linear dose-response between approximately 1000 - 10000 μg (1 - 10 mg)/plate.

After confirming that the presumed mutagens were positive in the Ames test, an evaluation of their mutagenic activity using different combinations was conducted. The experiments conducted involved the following:

- a) The selected mutagens were mixed in pairs over their positive concentration range and the results compared to the value expected from summing the individual responses.
- b) The four selected mutagens were mixed over their positive concentration range and the results evaluated for additivity.
- c) The "cocktail mix" was evaluated by testing the 4 mutagens, 16 non-mutagens and the full mixture at the same dose levels using TA-98 and TA-100 in both the presence and absence of metabolic activation.

The questions to be answered were:

- 1) Is mutagenic activity additive?
- 2) Is toxicity additive?
- 3) How do different mutagens (direct and indirect acting) behave in combination?

The tests for individual responses and combination responses were conducted at the same time. Each compound was tested over its positive dose range. The testing was conducted in triplicate at each dose and the average net revertants per concentration was calculated. This value is obtained when the average control value determined for the tester strain is subtracted from the average of the three replicates at each concentration tested.

Figures 1 - 8 show the mutagen combinations used and the results obtained in bar chart form. These experimental data will be discussed during the paper presentation.

CHEMICAL TESTING

Stock solutions of the individual chemicals were prepared by weighing known amounts of each and dissolving in a suitable solvent. Preliminary chemical data were obtained by direct injection of suitable aliquots (1-3 μ L) of the individual compounds initially, and then of a combined mixture of the compounds into a gas chromatograph/mass spectrometric (GC/MS) system. The system used was:

HP Model 5790A series capillary GC with an HP 5970A Mass Selective Detector. The data system was an HP ChemStation with an HP 9816S computer and HP 9133XV disc drive printer.

Instrument Parameters

(i) GC Conditions

Column	: 30 m x 0.25 mm fused silica column (SP B5)
Injection	: 250 ^o C
GC/MS Interface	: 250 ^o C
Column Program	: 50 ^o C for 1 min; 10 ^o C/min to 275 ^o C
Carrier	: He at 1.5 mL/min

(ii) MS Conditions

Source	: Electron Impact with 70 eV
Electron Multiplier	: 2000-2400 volts
Scan	: 380 amu/sec; 4 samples per 0.1 amu
Mass Range Scanned	: 35-400 amu

Data was obtained for all the compounds using both total scan and selected ion monitoring procedures. Either or both methods will be used for identification and quantitation during the validation of extraction-/concentration techniques for recovery of the "cocktail mix" chemicals from groundwater and solid matrix materials. In Table IV the compounds are listed together with their respective retention times and the major ions (m/e) present in their mass spectra.

TABLE I

ORGANIC COMPOUNDS SELECTED for "COCKTAIL MIX"
to be USED in VALIDATION STUDIES for
EXTRACTION/CONCENTRATION PROCEDURES for
the ISOLATION OF ORGANIC COMPOUNDS from GROUNDWATER
and SOLID MATRIX MATERIAL

● Alcohols (none)	
● Aldehydes (none)	
● Aliphatics	* 1,2-dibromoethane 1,2-dichloropropane
● Amines	* β-naphthylamine 3,4-dichloroaniline
● Aromatics	o-xylene 1,2-dichlorobenzene Hexachlorobenzene 4,4-dichlorobiphenyl
● Esters (none)	
● Ethers/Ketones	Methyl isobutyl ketone
● Carboxylic Acids	Isobutyric acid
● Phenols	o-cresol 2,4,6-trichlorophenol 4-nitrophenol
● Nitrogen Compound	Quinoline * 2-nitrofluorene
● Polycyclic Aromatic	Methyl naphthalene (α or β) Fluoranthene * Benzo[a]pyrene 9,10-dimethylanthracene
● Sulphur Compound	Benzothiozole
● Pesticides (none)	
● Phthalates (none)	

* known mutagen

TABLE II

Compound	Toxicity occurs between µg per plate		
Benzo[a]Pyrene	100	500	µg
2-Nitrofluorene	10	50	µg
Methyl-isobutyl-ketone	4000	8000	µg
Fluoranthene	5	10	µg
9,10-Dimethylantracene	Not toxic	500	µg
4,4-Dichlorobiphenyl	100	500	µg
4-Nitrophenol	500	1000	µg
2,4,6-Trichlorophenol	100	500	µg
Hexachlorobenzene	Slight toxicity	50	µg
β-Naphthylamine	Slight toxicity	1000	µg
1,2-Dichloropropane	5780	11560	µg
1,2-Dibromoethane	10900	21800	µg
2-Methylnaphthalene	50	100	µg
3,4-Dichloroaniline	100	500	µg
Benzothiazole	1238	6190	µg
Quinoline	1093	5465	µg
Isobutyric acid	950	4750	µg
o-xylene	449	897	µg
o-cresol	1048	5240	µg
1,2-Dichlorobenzene	130	515	µg

The compounds (9,10-Dimethylantracene 500 µg and Hexachlorobenzene 50 µg) were not toxic at their solubility limit. Two compounds (2-Nitrofluorene and 1,2-Dibromoethane) were mutagenic for tester strain TA-100 as tested in the toxicity assay.

TABLE III

Mutagenic Responses to Salmonella Tester Strains

Compound	TA-98	TA-98	TA-100	TA-100
	W/O S-9	WS-9	W/O S-9	WS-9
DBE	Neg	Neg	Pos	Pos
2NF	Pos	Pos	Pos	Pos
BNA	Neg	Neg	Neg	Pos
B[a]P	Neg	Neg	Neg	Pos

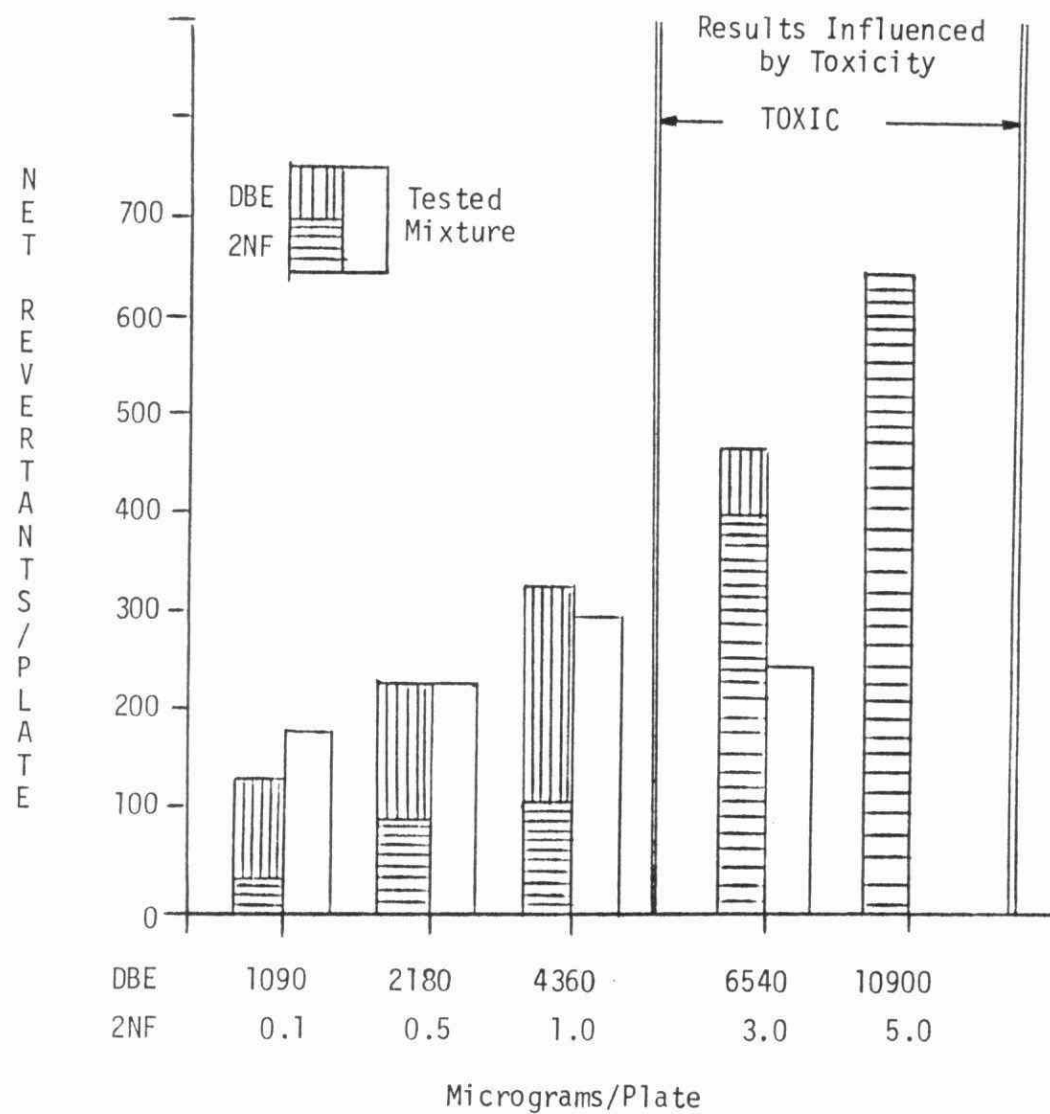
TABLE IV

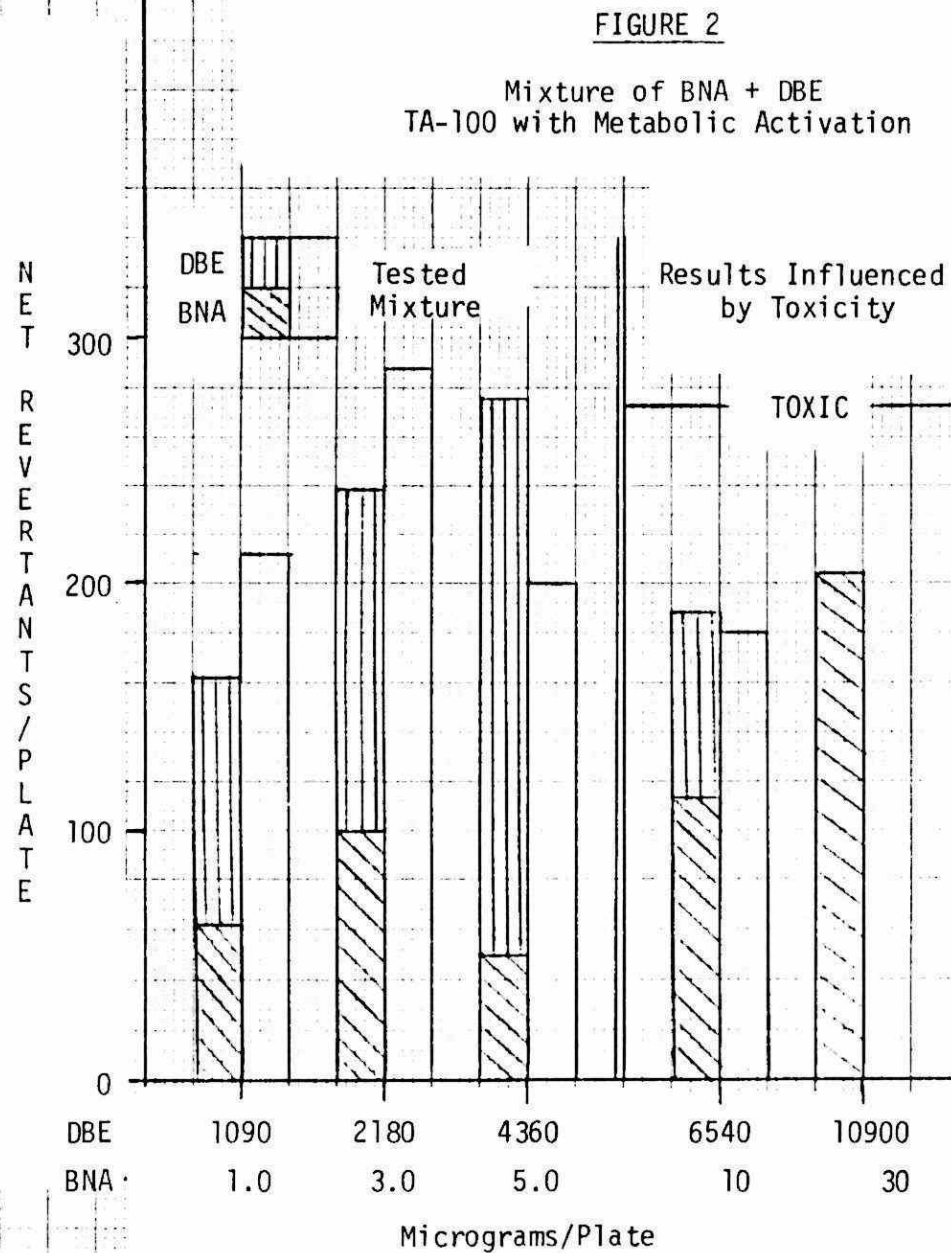
Gas Chromatographic and Mass Spectral
Data for Chemicals in "Cocktail Mix"

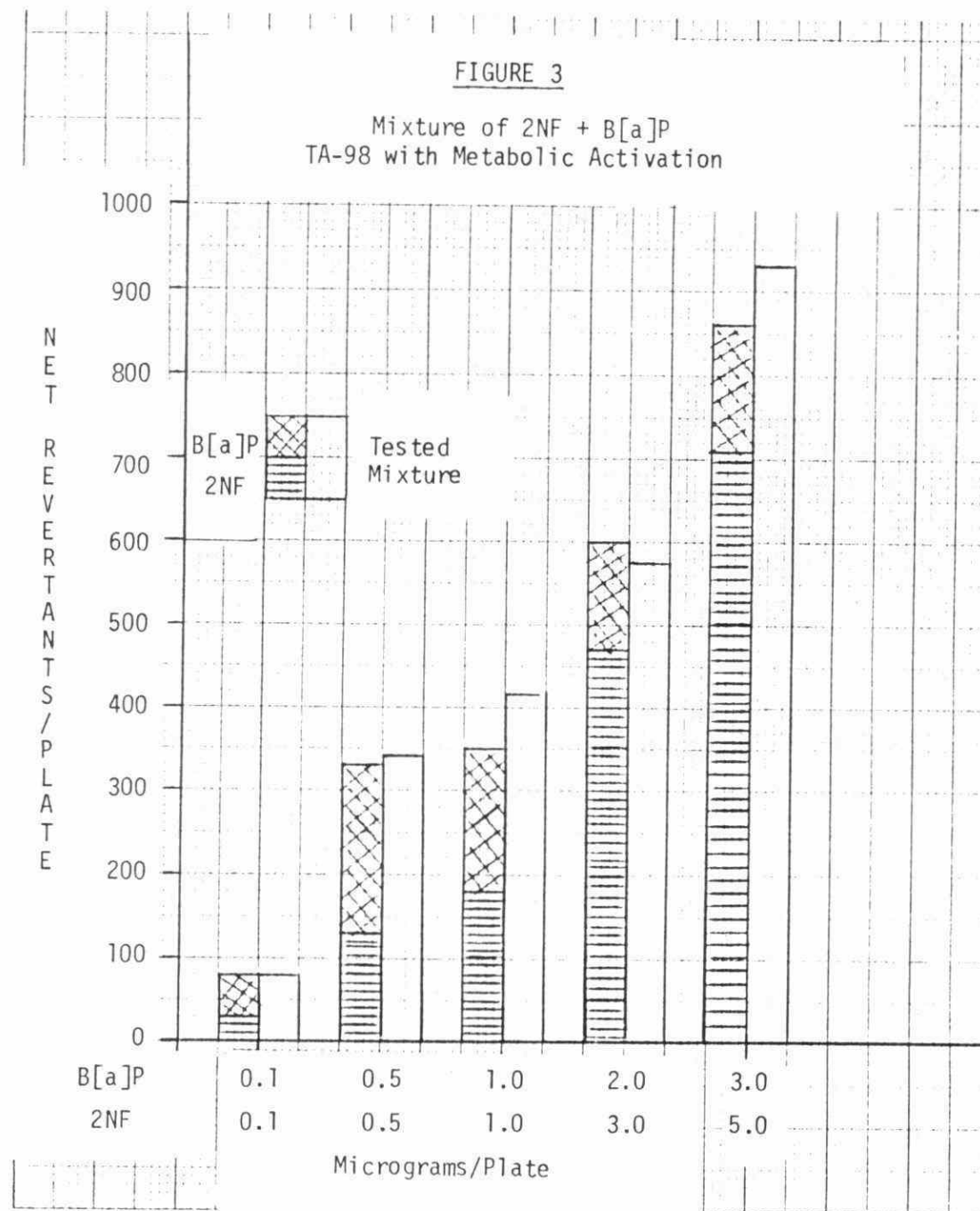
Compound	Retention Time (min)	Base Peak in Mass Spectrum
1,2-dibromoethane	2.35	27
o-xylene	3.53	91
1,2-dichlorobenzene	5.84	146
o-cresol	6.27	108
Benzothiazole	8.99	135
Quinoline	9.20	129
2-methylnaphthalene	10.20	142
2,4,6-trichlorophenol	11.07	196
3,4-dichloroaniline	12.10	161
4-nitrophenol	13.57	139
β -naphthylamine	13.73	143
Hexachlorobenzene	15.81	284
4,4'-dichlorobiphenyl	16.72	222
Fluoranthene	19.74	202
2-nitrofluorene	20.26	165
9,10-dimethylanthracene	20.51	206
Benzo[a]pyrene	33.26	252

FIGURE 1

Mixture of 2NF + DBE
TA-100 without Metabolic Activation







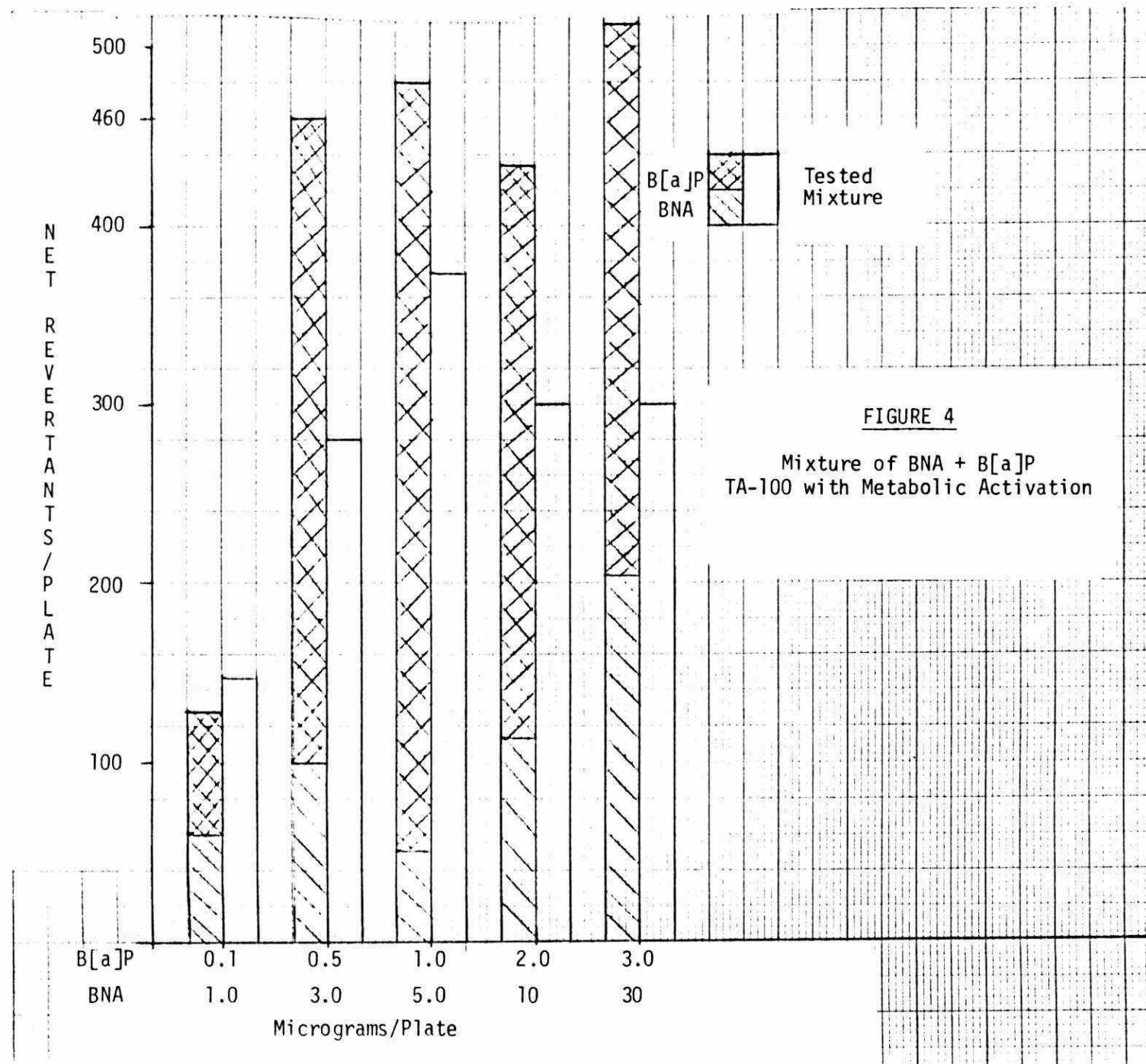


FIGURE 5

Mixture of 2NF + DBE + BNA + B[a]P
TA-98 without Metabolic Activation

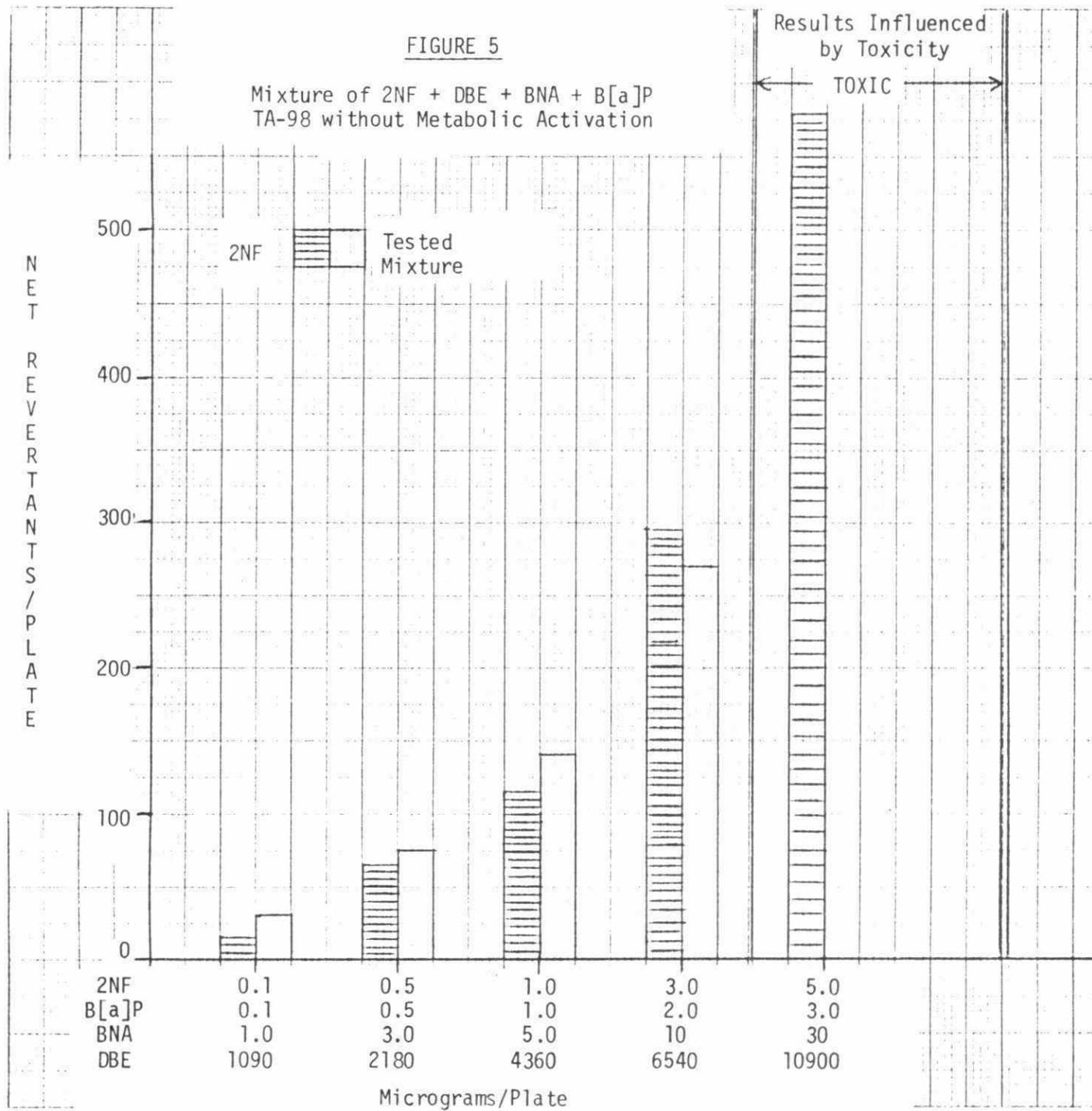


FIGURE 6

Mixture of 2NF + DBE + BNA + B[a]P
TA-98 with Metabolic Activation

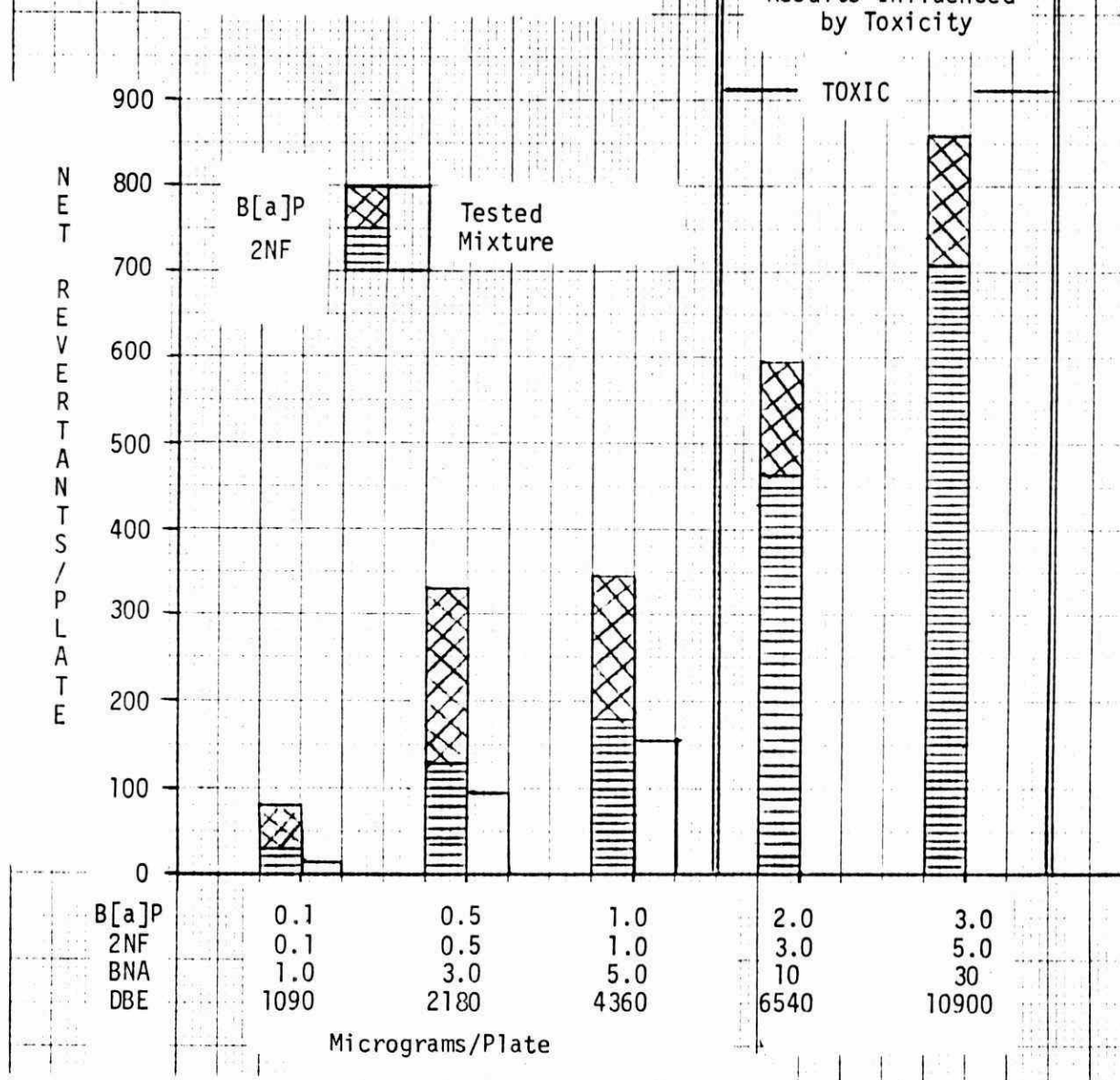


FIGURE 7

Mixture of 2NF + DBE + BNA + B[a]P
TA-100 without Metabolic Activation

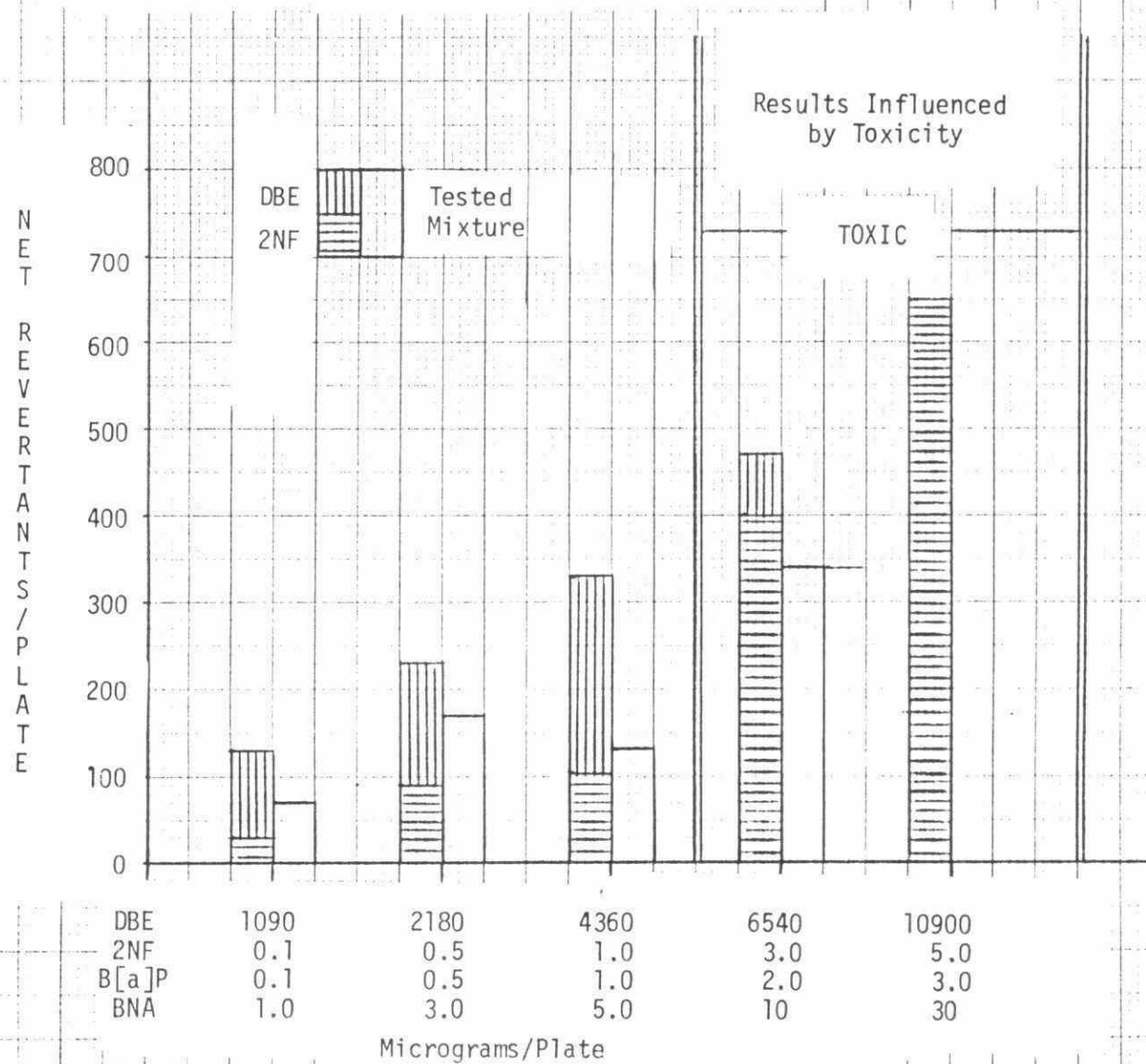
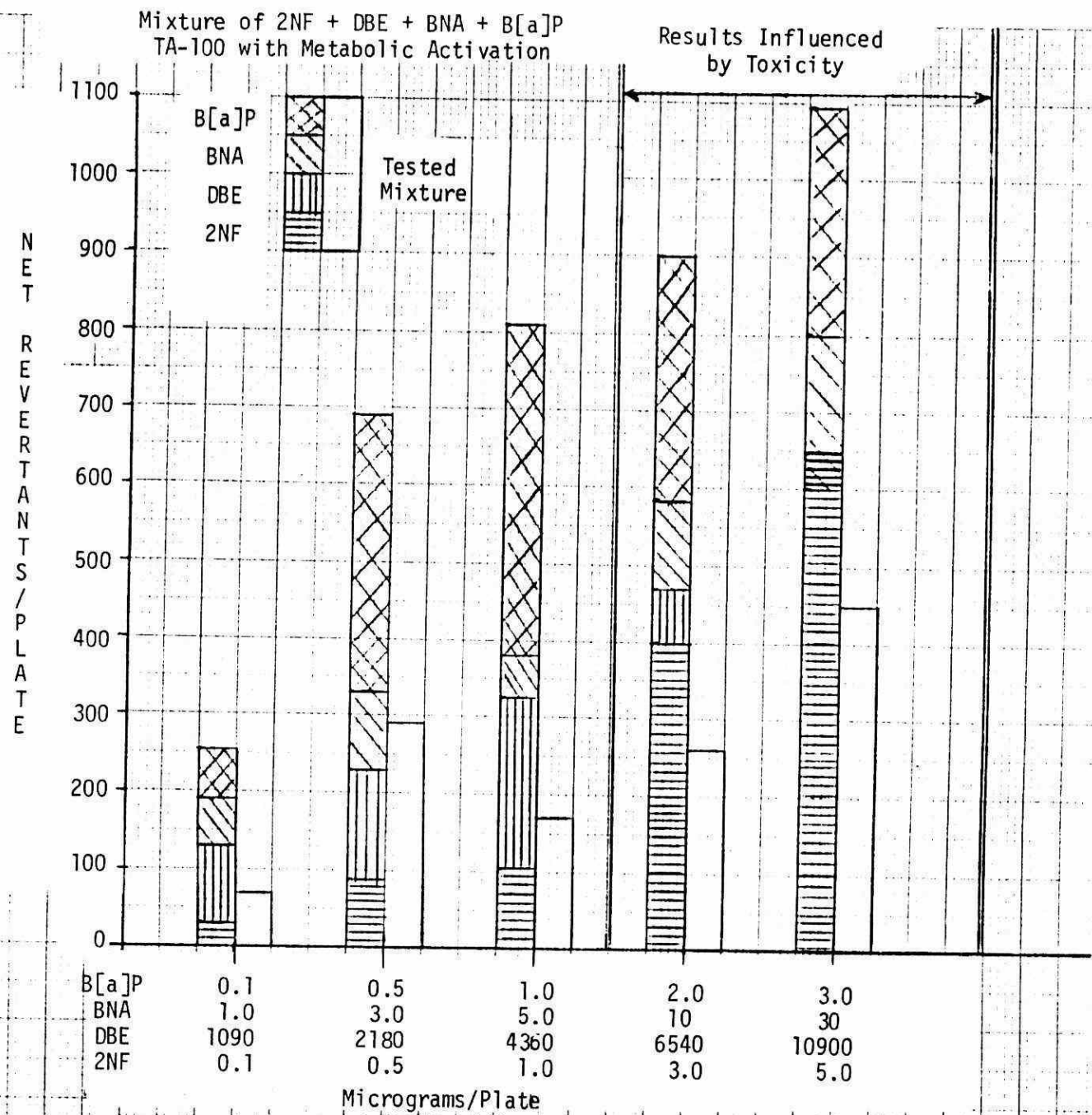
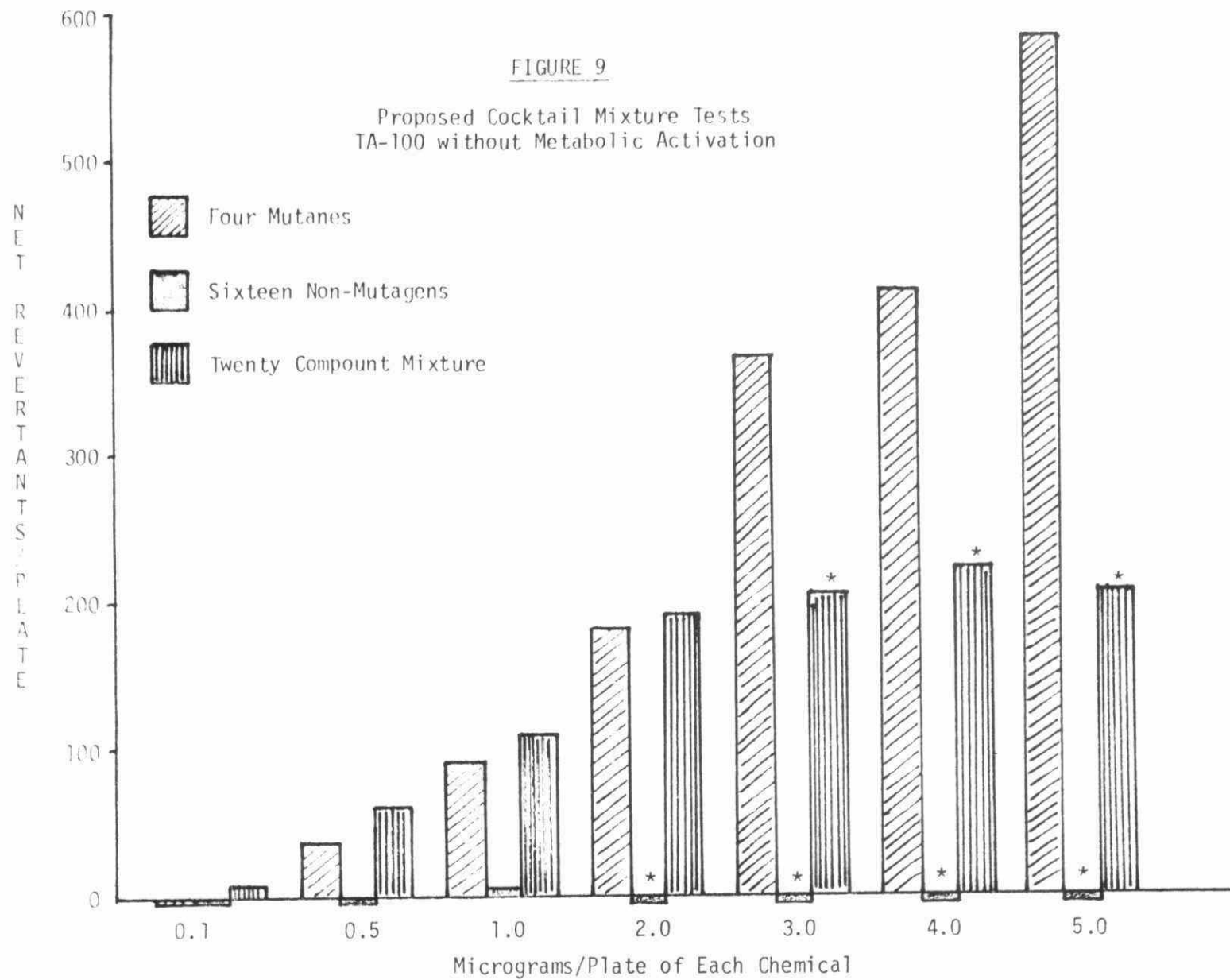


FIGURE 8

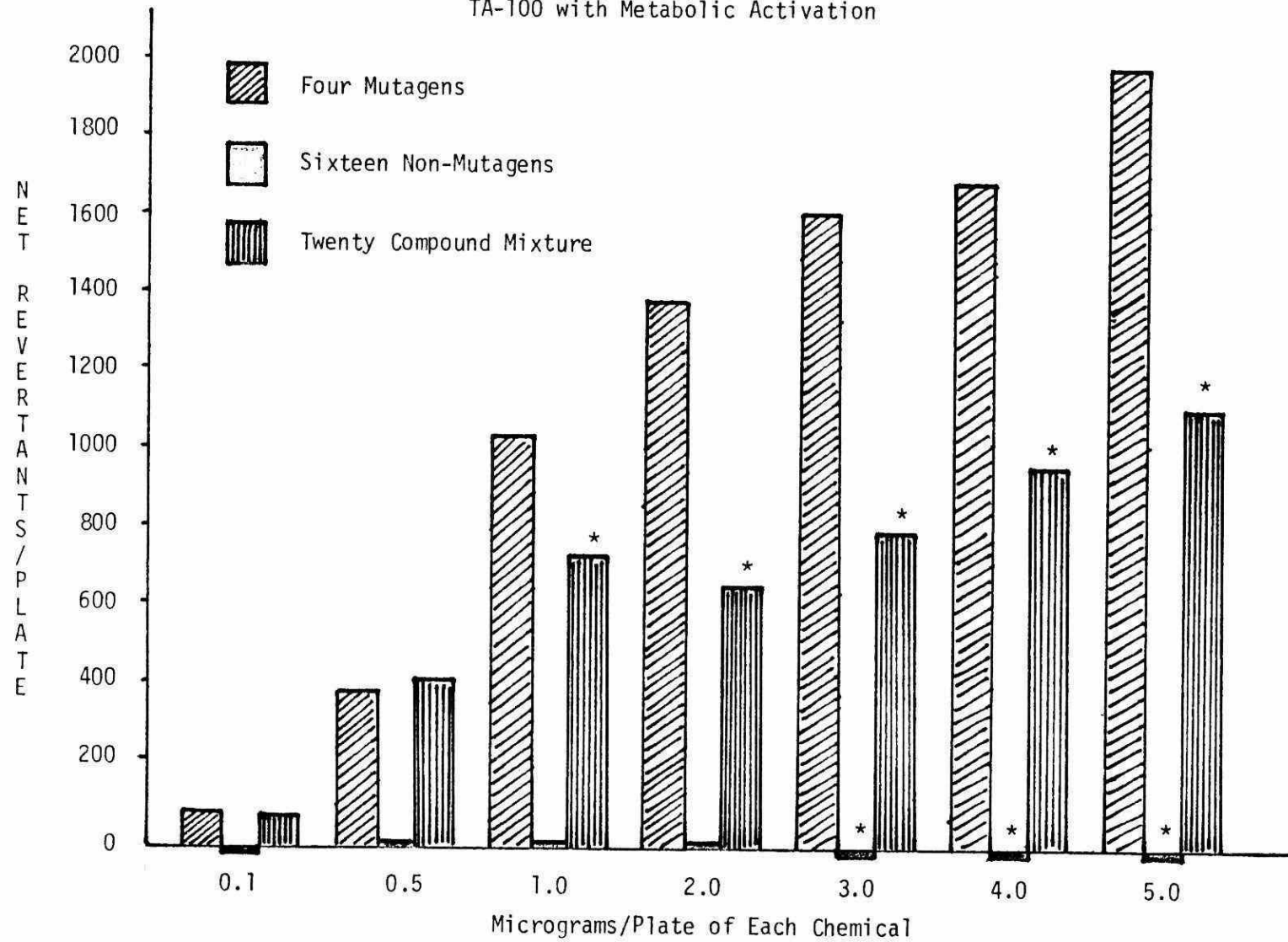




* slight toxicity

FIGURE 10

Proposed Cocktail Mixture Tests
TA-100 with Metabolic Activation



* slight toxicity

SYNTHESIS OF OXYGEN AND SULPHUR
POLYNUCLEAR AROMATIC HYDROCARBONS OF INTEREST IN
ENVIRONMENTAL POLLUTION

(Prepared for 1986 Technology Transfer Conference)

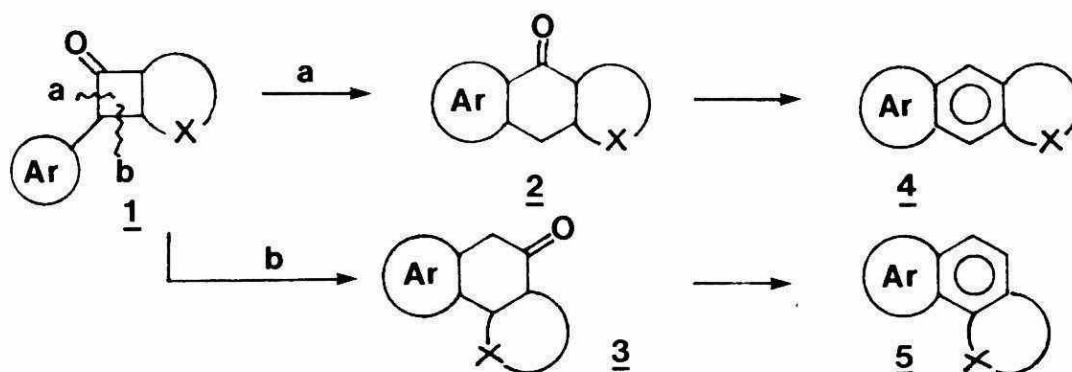
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The occurrence of polynuclear aromatic hydrocarbons (PAH's) in the environment and their toxicological properties has prompted wide studies in both the environmental and toxicology fields¹. Recently a number of cyclopentene annelated PAH's have been found to exhibit moderate to high levels of mutagenic activity². Of particular interest have been the derivatives of cyclopenta(c,d)pyrene, a non-bay region hydrocarbon shown to possess significant levels of activity in the Ames test. The metabolic activation of these derivatives takes place by way of a peripherally fused cyclopentene epoxide and subsequent binding of the epoxide to nucleophilic sites in cellular macromolecules³.

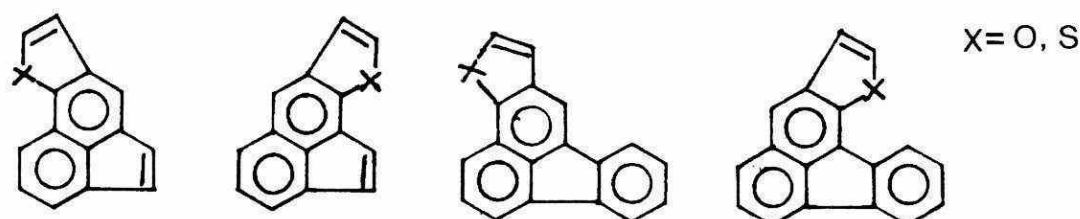
Moderate to strong mutagenic properties have also been associated with the seco derivatives aceanthrylene and acephenanthrylene⁴. In contrast to the carbocyclic systems relatively little is known about the biological properties of sulphur and oxygen heterocyclic analogues of mutagenic and carcinogenic PAH's primarily because of the lack of standards. In this study we report the synthesis of annelated benzofurans and benzothiophenes using two approaches which we have developed in our laboratory. The first approach involves the use of α -aryl substituted bicyclic cyclobutanones fused to a heterocyclic ring and the transformation of these intermediates by regiospecific ring-opening reactions to linear or angular fused PAH's⁵. The second approach involves the **S** or **O** cyclization of α -aryl or diarylmethylcations directly bonded to a thiocarbonyl or carbonyl group.

Regioselective ring-opening of bicyclic ketones **1** proceeds by α -cleavage

in the presence of base (path a). The intermediate ester/acid can then be

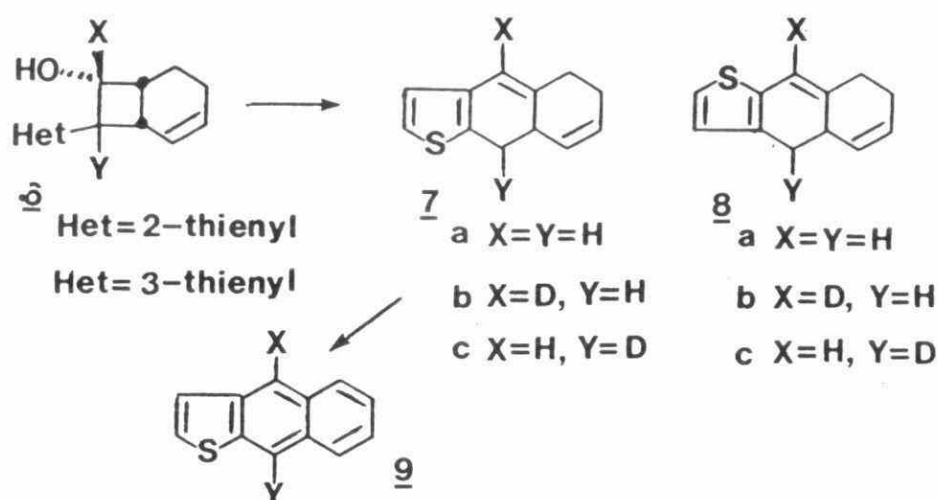


cyclized by standard methods to give ketone **2** which when reduced with LAH and dehydrated/dehydrogenated is transformed to the linear fused PAH **4**. Alternatively, by treatment of **1** with acid under a variety of conditions the isomeric ketone **3** is formed (path b) and can be converted to the angular fused heteroaromatic system **5**. Using this approach the following heterocyclic PAH's have been synthesized starting from the appropriately substituted bicyclic ketones **1**:

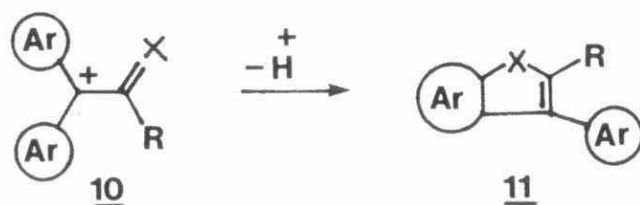


In an effort to prepare bis heterocyclic PAH's we considered the possibility of using bicyclic ketones **1** in which the α -aryl substituent was part of another heterocycle. The initial studies were done on α -2 and α -3-thienyl derivatives of **1** in which the X-fused ring was a cyclohexene. The analogous reactions "a" and "b" did not proceed as efficiently as in the case of the carbocyclic α -aryl substituted ketones **1**. In view of our previous studies⁶ using cyclobutanols, analogous acid transformations and subsequent structural modifications led to the preparation of angular fused PAH's **5**. We investigated the acid rearrangements of derivatives **6**. These were efficiently transformed to the unexpected linear tetrahydro derivatives **7** and **8** which were dehydrogenated to the linear PAH **9**. The mechanism for the

formation of the unexpected linear derivatives **8** and **9** was elucidated by studies with specifically substituted deuterated derivatives of **6**⁷.



The second approach was based on work we carried out using diarylmethyl cations substituted with carbonyl groups⁸. These intermediates undergo fluorene cyclization in the case of the diaryl derivatives or oxygen cyclization depending on the nature of the carbonyl group (ketone or carboxylic acid derivative)⁹. Oxygen cyclization gives rise to annelated



benzofurans **11** ($X=O$). We have recently observed that diarylmethyl cations in which the cation is substituted with a thioformamide group rearrange exclusively to annelated benzothiophenes **11** ($X=S$). The mechanism for these transformations has been partially resolved by studies with unsymmetrically substituted diarylmethyl cations **10**. Such cations **10** are routinely generated from the corresponding alcohols.

In conclusion, two independent methods have been developed in our labs

for the preparation of annelated benzofurans and benzothiophenes. By appropriate substitution of the precursors a wide variety of these heterocyclic PAH's can be obtained.

We would like to thank the Ministry of the Environment, Province of Ontario, ARB for their generous support of this project.

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DEVELOPMENT OF A ROBOTIC WORKSTATION
FOR THE ROUTINE ANALYSIS OF
ORGANICS IN BIOLOGICAL TISSUES
USING ACID DIGESTION AND SOLVENT EXTRACTION METHOD

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1.0 INTRODUCTION

The increasing concern over the presence of persistent toxic chemicals in the food chain has led to an increase in the number of analyses that must be performed annually. However, due to the current fiscal climate, available resources have not kept pace with this increase in the workload.

As is the case with most types of analytical work, the sample preparation step is usually time consuming and prone to error. To alleviate this bottle-neck, a robotic sample preparation system is being developed.

The system being used to implement this procedure is the Zymate II Laboratory Automation System, manufactured by Zymark Corporation of Hopkington, Mass.

The system design objectives are as follows:

- i. Improved precision and accuracy of results.
- ii. Cost savings in materials such as solvents.
- iii. Reduced operator exposure to hazardous chemicals.
- iv. Improved data reduction and reporting.
- v. User friendliness.

The development of this system involved precise specification of a robotic method based on a manual procedure, the acquisition and modification of the hardware required by the method, the design and testing of software, and validation of the completed system.

This paper presents the progress of this project at the time of writing. Further evaluation will be undertaken to assure that all design requirements are met.

2.0 SYSTEM HARDWARE

After evaluation of the manual method, the hardware components decreased necessary to convert to an automated method were as follows:

- (i) Robot arm and Controller
- ii) IBM-compatible PC
- iii) Master Laboratory Station and Remote Pipetting Kit
- iv) Electronic Balance
- v) Sample Conditioning Station (Linear Shaker)
- vi) Capping Station
- vii) Centrifugation Station
- viii) Drying/Neutralization (Prep_Sep) Station
- ix) Waste/Rinse Station
- x) Assorted Racks
- xi) Printer

2a DESCRIPTION OF WORKSTATION COMPONENTS

a) Robot Module

The "robot" itself consists of an "arm" secured to a benchstop by a pedestal. This allows three degrees of freedom of motion. The arm is capable of holding a variety of "hands", which rotate about a wrist connection, giving one extra degree of freedom.

The range of motion in each direction is:

- (a) Vertical : 0-34 cm
- (b) Reach : 0-32 cm
- (c) Rotary : 0-376°

The arm's locomotive power is provided by a wire pulley system, driven by DC servomotors. Directional control is provided by programmed commands. Initial calibration of the arm at the factory provides a frame of reference, so the arm "knows" it's current position. To go to a new position, the controller compares the current position to the desired position. This generates an error signal, which drives the servo's in such a way as to minimize the error signal.

There are four different types of hands available to this system:

- (a) General Purpose Hand
- (b) Syringe Hand
- (c) Dual Function
- (d) Blank Hand

In this application, the General Purpose Hand and the Blank Hand are used.

The General Purpose Hand consists of a teflon block housing a servomotor and has two "fingers" (grippers) attached. This hand is used for manipulation of glassware. The joint of the

hand and arm forms a "wrist" capable of rotation from -5 to 365° . In addition, the force with which the fingers grip an object can be adjusted from 0 to 200 units.

The arm and hand assemblies can also receive feedback from the external world in each of three axis:

ie: Vertical Force
Reach Force
Rotary Force

as well as Grip Force in the hand. This allows the arm to "sense" possible error conditions while performing operations on the bench.

The arm also allows different speed settings for each axis and can sense a collision, in which case it stops, and can only be restarted by operator intervention.

b) Master Laboratory Station and Remote Pipetting Kit

(i) The Master Laboratory Station consists of a housing containing three 10ml syringes, valving to control fluid flow, stepper motors to drive the plungers, and a dispenser nozzle mounted on a post.

The syringes can be programmed either independantly or jointly, to dispense/fill from a reservoir. Plunger speed can also vary to compensate for fluids of different viscosities.

The Dispenser nozzle contains separate outlets to avoid cross-contamination of liquids.

The syringes are programmed by "setting" the volume, speed and flow conditions necessary in a given operation.

Accuracy and precision for this station are quoted at 0.5% and 0.2% respectively, where volumes are greater than 20% of total syringe volume.

(ii) Remote Pipetting Kit

The remote Pipetting Kit allows pipetting/dispensing to vessels at fixed positions on the benchstop. It consists of a Blank Hand with a pipet-tip holder and pneumatic shucker, the parking station for this hand, and tubing for attachment to external compressed air, the MLS, solenoid operated valve, and a pipet-tip rack.

After attachment of this hand, programming is the same as for programming of any other robot moves. The pipet-shucking operation is driven from the Power and Event Controller, where actuation is by switch closure.

c) Power and Event Controller

The Power and Event Controller permits programmed operation of external devices. It provides the options listed in the following manufactures documentation.

In this application, it is used to discard pipet tips and operate vacuum for the Rinse/Waste station. The large number of control functions will permit future expansion to the system, should it be required.

The Power & Event Controller

The Power & Event Control module provides programmable control of laboratory devices external to the Zymate System. This module consists of:

- o 8 isolated relay contacts for device control
- o 8 logic inputs that sense high/low voltages or switch closures
- o 2 on/off 115 VAC power outlets
- o 1 variable AC power outlet
- o 1 analog/digital converter that accepts a 0 to 2V analog signal from devices monitoring outside factors
- o 3 power supplies (+5V, +12V, -12V) which provide additional sources of power for external devices

SPECIFICATIONS

SWITCHED OUTPUTS:

Relays

Reed Switch, Glass Enclosed

Contact Ratings

1/2 Amp. Max - DC or AC RMS

24 Volts Max - DC or AC RMS

SENSED INPUTS: (TTL Compatible)

Resistor

4.7K Pullup

Voltage, open circuit

5.0

Current, external switch closed, 1.0 ma

A/D INPUT:

Input Range

Ground to +2V

Converter

Increments

1 mV

Precision

+/- 0.1%

High Input Impedance

Sampling Time

1 sec

SWITCHED A.C. OUTPUTS:

At any one time, no more than 5 amps or 600 VA TOTAL may be drawn from all 3 AC outlets

VARIABLE A.C. OUTPUT:

At any one time, no more than 5 amps or 600 VA TOTAL may be drawn from all 3 AC outlets

POWER SUPPLIES

5V

1/2 A

+12V (adjustable: 2 to 12 V)

1/2 A (decreases as voltage decreases)

-12V (adjustable: -2 to -12 V)

1/2 A (decreases as voltage decreases)

d) Printer Module

The system permits operation of a printer to provide hard copies of data and programs. It can be programmed to be either enabled or disabled and provides essentially a line by line screen dump.

e) Balance Module

The balance used in this application is a Mettler PE200. The module consists of the Balance Interface Card, a 5-pin to 32-pin cable and a sample tube holder. The balance is capable of sending weight results to the Controller via the balance interface card, which is compatible with Mettler's CL interface and capable of receiving commands from the controller. It is a menu-driven programmable unit, where baud rate, parity and data transfer Mode may be set and stored. In addition, dampening functions may be set to compensate for vibration and time-averaged readings are also possible.

Operation is from the controller, where weight and tare commands are defined. Results are stored in arrays for use in calculation of masses.

f) Capping Station

The Capping Station consists of the capper (an enclosed motor with a set of jaws on the top surface), and a cap parking station, where bottle caps are temporarily held.

The Capper's operation must be carefully coordinated with that of the arm. During a cap operation, a cap is retrieved from the parking station and lowered onto the tube

while the capper rotates. Similarly, during an uncap operation, the tube is placed in the capper, the jaw grips the cap and the capper rotates in the opposite direction while the arm is incrementally raised.

The station can be programmed for

- (i) Capping Torque
- (ii) Number of Turns required to uncap/cap

Error routines are incorporated into the software to detect most common types of errors i.e. grip slipping.

g) Drying/Neutralization Station

This station consists of:

- (i) a prep-sep dispenser
- (ii) a prep-sep workstation

It is run from the PEC, where a switch closure will result in pressure being applied to a prep-sep containing a solvent aliquot. The prep-seps contain a layer of sodium bicarbonate (0.5g) and a layer of anhydrous sodium sulfate (0.5g). The pressure forces the solvent through the prep-sep, where the aliquot is dried and neutralized.

This workstation will save the expense of a powder pouring station and it's associated equipment as well as limited bench space and time during the running of the application.

h) Sample Conditioning Station

The Sample Conditioning Station consists of a linear shaker and two vertical, five position rack. The station is used for the agitation of samples during digestion and extraction, where the arm will load the tubes in a

horizontal position.

The shaker is capable of speeds from 1 - 100 units and always stops at the same position, to facilitate access by the robot arm during insertion/removal from the rack.

i) Waste/Rinse Station

The Waste/Rinse Station will be used to aspirate the fish/acid residue and unused solvent to a waste holding tank. It is run from the PEC, where switch closure results in vacuum being applied.

The Rinse part of this station consists of a Hi-Volume Dispensing Kit, which contains a high-speed peristaltic pump, tubing, valving, post and dispenser nozzles. When switched on, the tube will be washed out with water, supplied by the pump.

The Centrifuge Station

The Centrifuge Station adds automated centrifugation to the Zymate System. The Centrifuge Station offers a choice of two rotors: a 4 position rotor accepting tubes up to 50 ml in size; or, a 6 position rotor accepting tubes up to 15 ml in size. The Centrifuge Station operates at speeds between 300 and 3000 rpm with weights up to 100 grams per rotor position.

The Centrifuge Station will detect loads that are unbalanced, when the desired speed cannot be attained (within 50 rpm) or when its door is open. These conditions will cause a spin in progress to be aborted. Also, a spin will not be started with the door open unless the automatic door successfully closes.

There are a few sample load requirements in order to assure a successful centrifugation:

- o The rotor must never be run with buckets empty or other uneven (though balanced) loads.
- o All loads must balance within 1 gram.

This station provides programmable control of:

1. Rotor speed (up to 3000 rpm)
2. Spin time (up to 99.9 minutes)
3. Sensing of error conditions (out-of-balance, inability to reach speed, inability to find position, door opened during spin, door jammed)
4. Tube position

These programming controls may be set on a programming screen for direct operation of the Centrifuge Station. Or, command variables whose values are defined during the running of a program or in the Direct Control mode may be named.

2b OUTLINE OF SYSTEM CONTROLLER

a) Hardware

The Zymate II System employs several 16-bit chips. The main CPU is the Intel 8088, with an 8087 used as a math co-processor (the Fast Math upgrade from the Zymate I). Memory is 64K, with the operating system occupying 4K, leaving 60K for user applications. RAM in this system is non-volatile, battery-supported.

The Zymark System is very flexible due to the modular concept employed in it's design. In this design, each module (ie Capper, MLS) has it's own module card, where it's intelligence is contained on ROM chips. The module card is linked to it's module by a control cable, and to the controller by module support boards. Each module support board can hold up to five module cards, and there is room for five module support boards, hence, we may have up to 25 modules in a given application.

The System may be interfaced to an external computer by use of the Computer Interface Module. This module provides an RS232/423, ASCII, serial communications link between the controller and an external, more powerful laboratory computer. The interface parameters that may be set are:

- (i) Baud Rate : Up to 1200 Baud
- (ii) Character Length : 7 or 8 bits/character
- (iii) Stop Bits : one or two

This will allow the Computer Interface to "talk" to almost any PC we choose to use, providing it has a

communication board and uses the same RS 232 standard.

b) Software

The Zymate II System provides a menu-driven system for operator convenience.

Upon initialization of the system, the Controller polls the modules plugged into the module support boards, and configures the system accordingly. The initial menu is displayed, and gives one five choices:

1. Run Program
2. Edit Program
3. Bench and Module Set-up
4. System Management
5. Direct Control

1. Run Program: allows one to execute an existing EasyLab Program.
2. Edit Program: allows one to edit or create programs. This is a line-editor, sufficient for most purposes.
3. Bench and Module Setup: this selection displays the modules currently configured in the system. From this menu, we may enter a control "page" which gives direct control over that particular module.
Each module has a control page where defaults are set or changed. An example is the computer interface, where the baud rate, parity and stop bits may be set, depending on the characteristics of the external computer.
4. Systems Management: displays the status of the RAM batteries, amount of dictionary space left, and allows one to specify an autostart program.
5. Direct Control: allows the execution of programs, commands directly.

The Zymate II allows programs to be written in it's language called EasyLab. This is an interpreted language and has a flavor characteristic of BASIC. For example, the following constructs are allowed:

- (i) Arrays: numeric, single dimension only.
- (ii) DO Loops
- (iii) GOTO's
- (iv) IF conditions THEN statements : but no ELSE construct.
- (v) Real Math: 6 Significant figures.
- (vi) Standard Mathematical Functions : SIN, COS, ATAN, SQRT, LOG 10

EasyLab allows any user-defined variables to be globally available to any sub-routine called from within any program. The maximum number of levels of calls is limited to 7. Text processing is very limited, as there is only one type of PRINT statement.

To overcome the programming limitations of Easy Lab and the Controller, an external computer will be linked to the controller via the computer interface. With this configuration, a much more "intelligent" system can be designed, where the external computer can be executing a more complex program, and simply pass the appropriate commands to the controller. Likewise, the controller will pass information back to the computer, where decisions will be made.

The choice of a high-level language running on the external computer is essentially wide open, but QUICK-BASIC, a compiled version of BASIC, was recommended.

3.0 CHANGE TO MANUAL PROCEDURE

The currently used manual method is outlined in Appendix A.

In order to implement this method as a robotic procedure the following modifications were made:

- i. All manual extractions are carried out in 60 ml centrifuge tubes. Since the capacity of the centrifuge module is only for 50 ml tubes, all volumes had to be adjusted accordingly.
- ii. Robotic pipetting procedures are performed gravimetrically.
- iii. In the manual procedure, extract neutralization and drying steps are performed in Evlenmeyer flasks. To implement this step in the same manner would require too much bench space and additional glassware to be manipulated by the arm. In addition, the dispensing of powders are sufficiently time-consuming as to reduce system throughput. Our solution was to proceed with disposable columns packed with sodium bicarbonate and anhydrous sodium sulfate, which requires only one additional workstation.
- iv. The inclusion of a centrifuge module to break up emulsions and settle undigested fibrous material enables better separation of phases.
- v. The manual procedure requires the final extract to be volumetrically diluted to 100.00 mls. A one gram equivalent (or 1/5 of total volume) is removed for cleanup. Again, table space does not permit the required number of flasks. Instead, the total volume of extract is computed and a calculated volume is pipetted volumetrically.

4.0 UNIT OPERATIONS OF SYSTEM

The following is a detailed description of the robotic steps performed in the method:

- i. The initial step consists of taring a rack of capped, empty centrifuge tubes, which will be loaded with samples. This step is performed independantly of the main run sequence. All weights are stored in an array.
- ii. After taring, the operator removes the rack of tubes and loads each with 5.0 ± 0.1 g of wet fish tissue.

The tolerance of the weight must be adhered to, in order to ensure the centrifuge remains balanced. The loaded rack is replaced on the table and the run is initiated.

- iii. A sample tube is taken from the rack, and weighed to obtain actual sample weight.
- iv. The tube is uncapped, acid is dispensed, capped and weighed.
- v. The tube is then loaded into the linear shaker and digested for 1/2 hour.
- vi. After digestion, the tube is unloaded from the shaker, uncapped and extraction solvent added.
- vii. The tube is then capped, weighed (to obtain accurate weight of solvent added) and returned to the shaker.
- viii. The sample is then extracted for 1 hour.
- ix. After extraction, the tube is unloaded from the shaker. The arm then loads and balances the centrifuge. The sample is spun at 2500 rpm for 10 minutes to breakup any emulsion that may have formed and to settle out any undigestable fibre in the extract.
- x. The sample tube is then unloaded from the centrifuge and the balance tube replaced. The sample tube is then uncapped, placed on the balance and weighed (as a check). A holding tube covered with a gravity slip-cap is picked up from the rack, its cap removed and placed in the prep-sep work station. A prep-sep is then obtained from the dispenser and loaded into the prep-sep work station. The general purpose hand is then parked, the blank hand picked up and a 10 ml pipette tip is obtained from its rack.
- xi. To quantitatively pipette the extraction solvent, the arm positions the pipette tip over the tube on the balance and finds the surface of the liquid. Once this initial starting position is found, 95% of the solvent is drawn into the pipette and the arm then positions the pipette over the prep-sep, where the solvent is dispensed. The solvent is then slowly forced through the prep-sep, while the arm draws the remaining 5% slowly and accurately. The remaining solvent is then again dispensed and forced through the prep-sep.
- xii. The pipette tip is then discarded, and the blank hand is exchanged for the general purpose hand.

- xiii. The prep-sep is then also disgarded, the holding tube removed from the prep-sep work station covered with gravity slip-cap and returned to it's rack.
- xiv. The arms returns to the balance and removes the sample tube which at this point, is still uncapped.
- xv. A second aliquot of extraction solvent is added, for the second extraction; the tube is capped and weighed.
- xvi. Steps viii - xi are repeated for the second extraction.
- xvii. The pipette-tip is disgarded and the general purpose hand is picked up.
- xviii. The prep-sep is disgarded, the holding tube is returned to it's rack, the sample tube is removed from the balance and it's contents are aspirated to waste. It is then returned to the sample tube rack.
- xix. The general purpose hand is then parked and the pipetting hand is again picked up. Another pipette tip is attached.
- xx. The arm then positions itself over the holding tube, and since the volume of solvent is known, positions the tip slightly below the surface of the extract. The volume of a one gram equivalent is calculated and drawn.
- xxi. The arm then positions itself over the output tube, and the one gram equivalent is dispensed to the output tube. These tubes are not capped as the next step is the evaporation of the extract to dryness: (part of the subsequent manual cleanup).
- xxii. The pipette tip is then disgarded and the general purpose hand picked up. The holding tube is then gripped, it's slip-cap retrieved and returned to it's rack. The system is then ready to proceed with the next sample.

This outline lists the robotic steps performed on a sample in a sequential manner. However, to use each work station to it's greatest potential (i.e. minimize idle time) the system runs as a serialized procedure. This requires that modules such as the shaker and centrifuge operate independantly of the arm, so in effect the system idle time is minimized. This was accomplished

by fine-tuning the timing of each step so that synchronization between the arm and the modules was achieved.

In addition, at critical steps such as capping and getting a pipette tip, confirm operations are carried out to ensure the proceeding step was successful before the subsequent operation is carried out.

5.0 PROGRAMMING CONSIDERATIONS

As much as possible, error detection and correction routines have been built into the software. For example, due to variations in tube volume and sample size, the surface level of the extraction solvent will vary. Thus, the routine to find the initial starting point at which to begin a pipette operation is necessary. It was also discovered that the pipette tips are not perfectly straight and so may touch the wall of the tube. This results in an increasing weight reading during pipetting. The solution is to minutely shift the position of the arm in the horizontal plane until no more contact occurs.

The concurrency issue also arises, since under a serialized procedure samples will be at different stages of processing at the same time. This could lead to situations where different samples are competing for the same module. A good example is the balance, where one sample requires it for a pipette operation, another to obtain a sample weight and yet another to obtain a solvent weight.

The solution is to time balance the procedure so that each resource is available at the correct time. This involves the use

of expiration timers for each sample at each module and avoids the use of priority interrupts. This also guarantees an identical processing history for each sample, one of the major requirements of any automated procedure.

Another requirement of this system is the processing of sample data and text. To accomplish this, an IBM compatible P.C. was linked to the controller to provide:

- i. a menu-driven user interface to the system.
- ii. expanded data storage and processing capabilities to the system.
- iii. enhanced error-recovery.
- iv. uploading of data to other systems.

This way the controller functions as a transparent low-level module, while the P.C. functions as a high-level controller, responsible for data reductions, file manipulation and repeat production.

6.0 ESTIMATED SAMPLE THROUGHPUT

The breakdown of robotic moves and times is given in the Table below:

<u>Operations</u>		<u># Moves</u>	<u>Time</u>
i.	Get tube from Sample Rack	8	0.5 hr.
ii.	Weigh Tube	8	
iii.	Uncap Tube	16	
iv.	Add Acid	8	
v.	Weigh Tube	8	
vi.	Cap Tube	16	
vii.	Load Shaker	8	
viii.	Agitate Sample		
ix.	Unload Shaker	8	
x.	Uncap Tube	17	
xi.	Add Solvent	8	1.0 hr.
xii.	Weigh Tube	8	
xiii.	Cap Tube	16	
xiv.	Load Shaker	8	
xv.	Extract Sample		
xvi.	Unload Shaker	8	
xvii.	Load and Balance Centrifuge	26	

xxviii. Centrifuge Sample		0.033 hr.
xxix. Unload and Balance Centrifuge	26	
xx. Uncap Tube	16	
xxi. Weigh Tube	8	
xxii. Pipette Solvent	45	
Quantitatively		
xxiii. Repeat (xi - xxii)	169	
xxiv. Pipette One Gram Equivalent	18	
xxv. Aspirate Tubes	6	
xxvi. Cap Sample Tube	16	
xxvii. Return Tube to Sample Rack	8	
Total # Robotic Moves	483	2.56 hr. (non Robotic Time)

Since the time per Robotic Move is 30 sec., therefore
Robotic Time = 1,449 sec. = 24.15 min.

Since by definition the rate limiting element in an application is the greater of the robotic time or the analysis time, the robotic time is the rate limiting element. However, since the times are distributed as follows:

Total Robotic Time = 24.15 minutes
Analysis Time = 0
Digestion Time = 30 minutes
Extraction Time = 60 minutes

This does not lead to a straight forward serialization, due mostly to the long extraction times. Hence, time balancing of the procedure must be employed where the rate limiting time is found which is an even divisor into all times.

This involves building idle time into the system:

Total Robotic Time = 24.15 minutes
Analysis Time = 0
Digestive Time = 30 minutes
Extraction Time = 60 minutes
Idle Time = 5.85 minutes

Now the Sample Output Rate can be calculated where:

Sample Output Rate = # samples that can be processed/hour
= 60/RLE (in minutes)
= 60/30 = 2 samples/hr.
= 48 samples/day.

7.0 FUTURE EXTENSIONS

- i. The extension of this application to the clean up and analysis stages. Preliminary considerations show that extraction columns to selectively bind the organics in question may be used to bypass Fluorasil cleanup.
- ii. Due to the highly modular design of both hardware and software, this method may be modified with relative ease to other applications of similar scope. Once table layout for additional work stations required by a new application is performed software modification to drive the new method could be implemented and tested. Then the P.C. would contain a library of low-level routines, that could be called from any new application program.

APPENDIX A

5.3. Extraction Procedure - Fish

Most samples arrive for analysis as frozen foil wrapped fillets. Treat large whole fish individually. Wash off adhering mucous and pat dry with paper towel. Measure length to 0.1 cm and weigh to 0.1 g. Cut fish posteriorly along dorsal surface using stainless steel knife. Note sex. Strip epaxial musculature (the muscle above lateral line) from skin and place about 50 g in stainless steel cup and grind for 2 minutes with mechanical homogenizer (Virtis). Using a stainless steel spatula, transfer to aluminum foil cup, cover and designate laboratory number. Store in freezer (-40°C) until analysis.

In the case of small fish, such as minnows, the whole fish is homogenized. Smelt are headed, tailed, gutted and ground.

- 5.3.1. Using stainless steel spatula mix previously ground fish tissue in aluminum foil cup, to ensure homogeneity. Frozen samples should first be thawed for 3-4 hours.
- 5.3.2. Weigh 5 gm (± 0.1 gm) of sample into a previously tared centrifuge tube and record sample number and exact weight.
5 gm is the optimum sample size, smaller samples to a limit of 1 gm can be used when necessary. Maximum sample size is 7 gm.
- 5.3.3. Add 40 ml of concentrated Hydrochloric acid to the fish tissue in the tube and seal using teflon lined cap.
- 5.3.4. Affix tubes to rotary making tumbler ensure caps are securely sealed.
- 5.3.5. Operate tumbler at 30-45 rpm for 1 hour to ensure good dissolution of tissues.
- 5.3.6. Remove tubes from tumbler and add 25 ml 25% dichloromethane in Hexane (v/v). Replace caps securely.
- 5.3.7. Replace tubes on tumbler and extract at 30-45 rpm for 1 hour. Let stand if emulsion develops.
- 5.3.8. Remove upper solvent layer using pasteur pipette and bulb and transfer to erlenmeyer flask.

- 5.3.9. Repeat 5.3.6 - 5.3.8 with a second 25 ml 25% methylene chloride in Hexane (v/v).
- 5.3.10. Dilute acid layer voluminously with water and discard in suitable manner.
- 5.3.11. Add 1 - 2 gm powdered NaHCO_3 to erlenmeyer containing combined extracts and swirl gently to ensure neutralization.
- 5.3.12. Quantitatively transfer sample extracts from 125 ml erlenmeyer flask to a 100 ml volumetric using a filter funnel, glass fibre filter paper and sodium sulphate. Do not make sample up to the 100 ml mark until ready for cleanup.
- 5.3.13. Stopper flasks and store in appropriate cool, dark area for separation/cleanup.

Samples stored in this manner must be processed through cleanup and separation steps within 24 hours or irreversible precipitation of lipid and protein in the extract may occur and affect final analytical results.
- 5.3.14. Rinse all used glassware under running tap water then with reagent acetone and place in dishwasher or appropriate surfactant detergent bath.

5.4. Clean-up Procedure - Fish

The cleanup procedure used for fish, eliminates unwanted contaminants including lipids and the elution sequence used separates pesticides into three groups. In this procedure PCB's are separated (approximately 95% efficiency) from the DDT's in one step thus eliminating the need for further chromatographic techniques.

- 5.4.1. Make 100 ml volumetric flasks containing sample extract up to the mark. Pipette an aliquot containing equivalent of 1 g fish tissue (usually 20 ml) from volumetric flask. Transfer to graduated test tube and evaporate to oil residue on a Vortex evaporator. Add 1 ml hexanes.

NOTE: The natural oil in the sample acts as a keeper and allows this stage of evaporation to be taken to dryness (e.g. no solvent).

SECOND NOTE: The Florisil column described will handle up to 80 mg of oil. If the oil residue after evaporation is more than 0.1 ml, a volumetric dilution or alternate aliquot is required to decrease oil to within the 80 mg limit.

- 5.4.2. Assemble chromatographic columns by inserting Teflon plugs and a 0.5 cm glass wool plug in the bottom.
- 5.4.3. Remove Florisil from oven and pour enough to prepare 1 SPLIT CHECK COLUMN into a 100 ml beaker. Return Florisil bottle to oven.
- 5.4.4. Pour Florisil into column and tap gently to form a height of 24 cm.
- 5.4.5. Immediately apply split check sample (4.5.7.) in 1 ml hexanes to top of column.

NOTE: Split check comprised of waste fish extracts, fortified to 500 ng/ml op'DDT and 10 $\mu\text{g/ml}$ PCB. (Aroclor 1254:1260 = 4:1).

- 5.4.6. Allow sample to drain just to top of column and add 1.5 ml hexane to top.
- 5.4.7. Continue adding hexane, 1.5 ml at a time until the entire column is wetted.
- 5.4.8. Place reservoir on top of chromatographic column. Add 30 ml hexane to reservoir.
- 5.4.9. Collect a first fraction of 18 ml from the column and then collect 6-10 separate 2 ml fractions. Label tubes with corresponding volumes contained.
- 5.4.10. All fractions are analyzed as in 5.8. This SPLIT CHECK is performed every day to allow for changes in the Florisil activity caused by humidity fluctuation. It must be done to ensure adequate PCB/DDT separation. Compare fractions to op'DDT standard. The split is taken at the first major appearance of op'DDT.
- 5.4.11. Remove Florisil from storage oven and pour enough for 4 columns (approximately 40 ml) into a 100 ml beaker and return Florisil bottle to oven.
- 5.4.12. Pour Florisil from beaker into columns while tamping column to form a 24 cm bed.
- 5.4.13. Place 50 ml centrifuge tube under each column.
- 5.4.14. Immediately apply the 1 ml sample extracts (5.4.1.) to the top of the columns using a Pasteur pipette and rubber bulb.
- 5.4.15. Allow sample to drain onto column until it reaches the top of the Florisil.
- 5.4.16. Rinse sample tube with two 0.5 ml aliquots of hexane and apply these to the column one at a time as above.
- 5.4.17. Add hexane 1.5 ml at a time until entire column is wetted.
- 5.4.18. Place reservoir on top of column and add 30 ml of hexane to reservoir.
- 5.4.19. Collect hexane in 50 ml centrifuge tube to the volume determined in the split check procedure. This is fraction A1.
- 5.4.20. Turn off stopcock and decant excess hexane off top of column into waste receptacle.
- 5.4.21. Remove any remaining hexane using Pasteur pipette.
- 5.4.22. Add 10 ml 25% v/v dichloromethane solution (reagent 4.5.3.). Place 15 ml centrifuge tube under each column.
- 5.4.23. Open stopcock and collect 15 ml. This is fraction A2.
- 5.4.24. Before solvent level reaches top of Florisil bed, remove fraction A2 tube, insert a 15 ml tube, add 10 ml 25% diethyl ether dichloromethane to reservoir. Collect all solvent until column stops dripping. This is fraction A3.
- 5.4.25. Add 2 ml iso-octane to each extract and insert fraction tubes in Buchler Vortex Evaporator as in 5.2.14 and evaporate to 2 ml.

NOTE: Do not let samples evaporate to dryness or serious pesticide losses occur.

- 5.4.26. Make up to 5 ml in iso-octane.
5.4.27. Analyze samples according to 5.8.

<u>FRACTION A1</u> contains:	PCB HCB Heptachlor Octachlorostyrene	pp'DDE Mirex Aldrin
<u>FRACTION A2</u> contains:	α BHC γ BHC (Lindane) β BHC α Chlordane γ Chlordane	o,p'-DDT p,p'-DDD- p,p'-DDT Toxaphene
<u>FRACTION A3</u> contains:	Oxychlordane Heptachlor Epoxide Thiodan I, II and Sulphate Dieldrin Endrin DMDT	

- 5.4.28. Rinse all used glassware under running warm water and rinse with reagent acetone. Place in dishwasher or appropriate surfactant detergent bath.

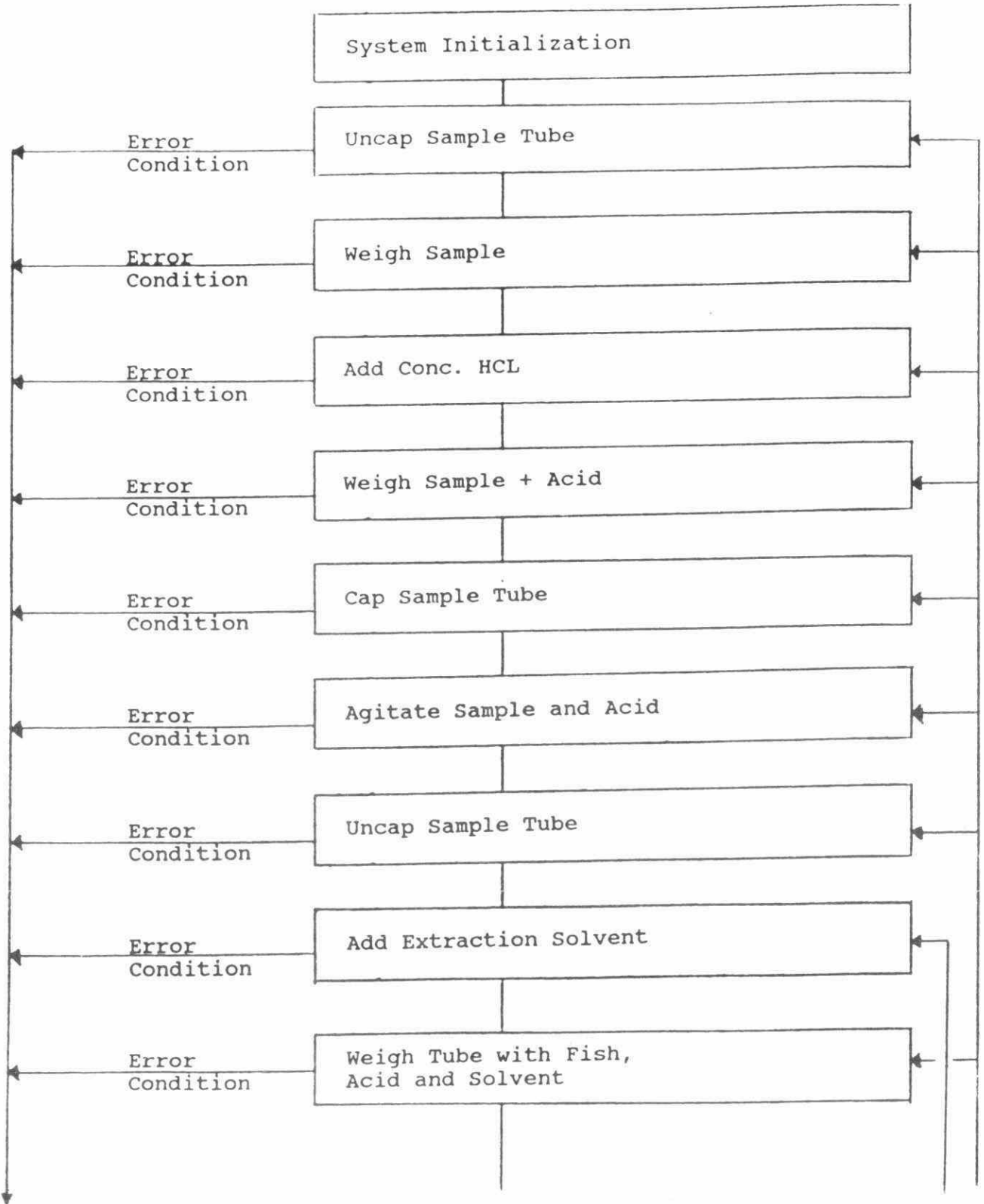
5.5. Extraction Procedure - Soils and Sediments (and Vegetation)

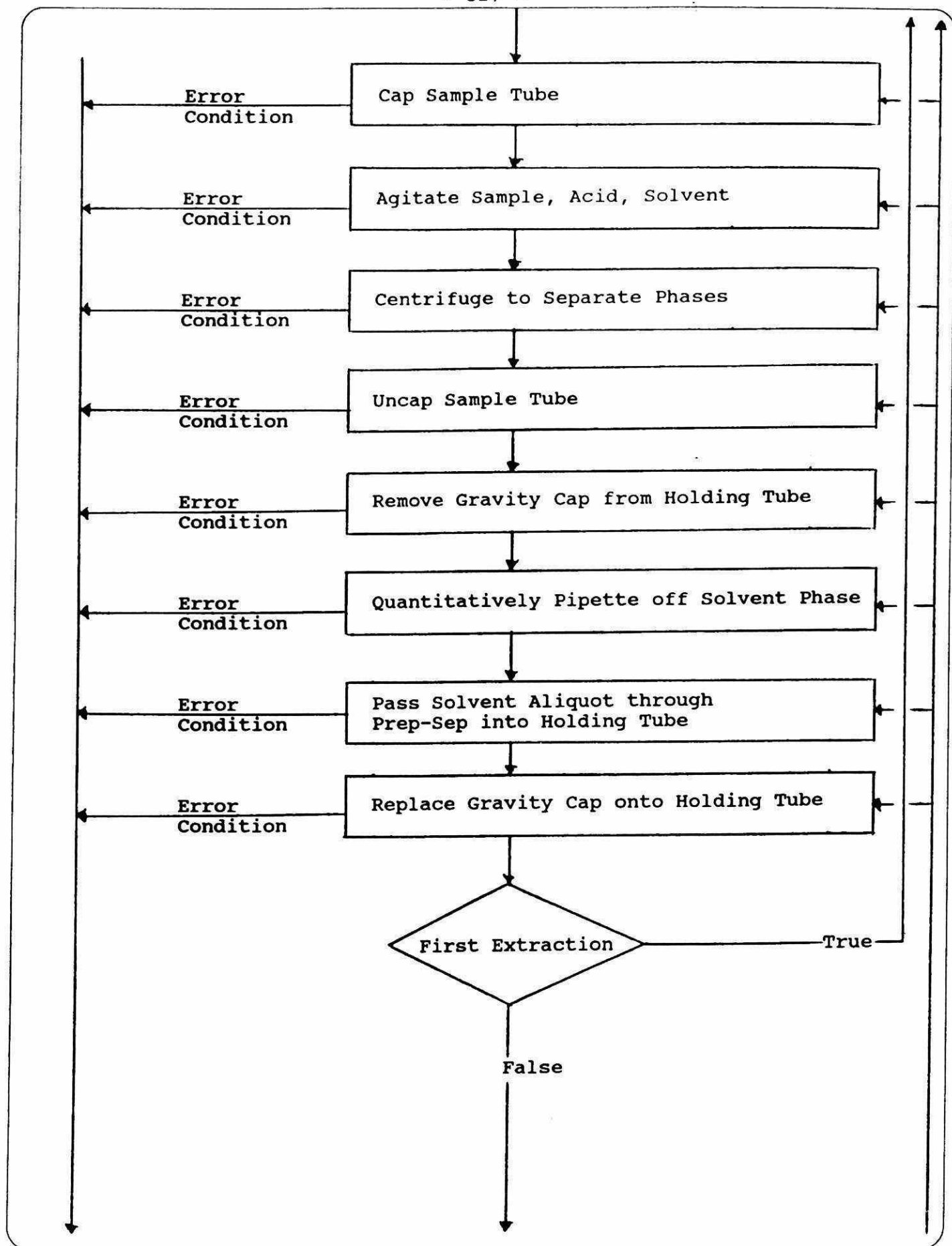
All sediment samples should arrive in the laboratory in solvent rinsed glass jars with foil-lined caps. Possible contamination causing unreliable results may arise from other containers. Although this procedure refers only to soils and sediment. Vegetation samples may also be analyzed according to this method by replacing the Sonifier extractor with a Polytron unit.

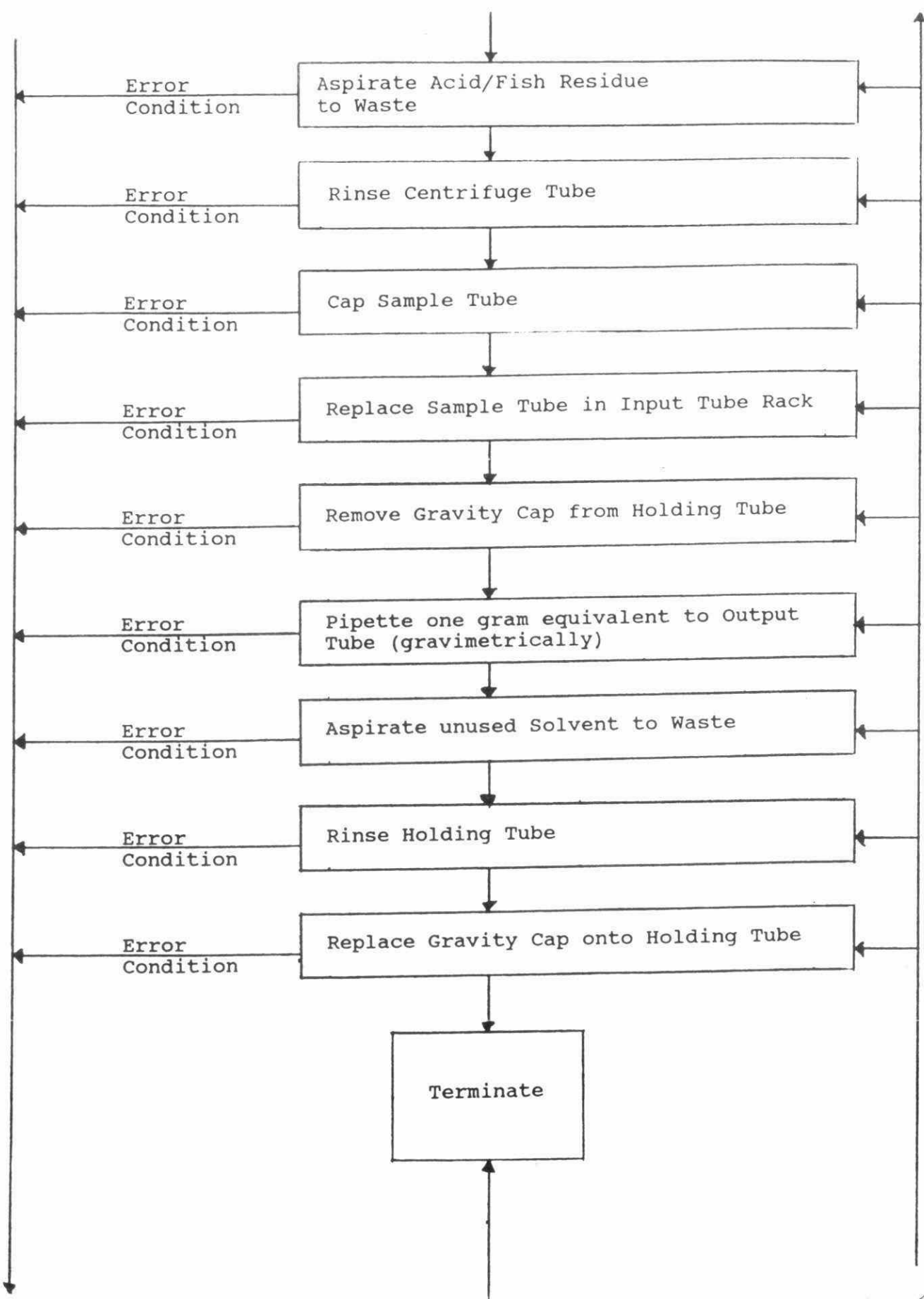
- 5.5.1. Determine water content of soil or sediment (see Moisture Determination section 5.7.).
- 5.5.2. Weigh appropriate amount (± 1 g) of wet sediment into 300 ml Berzelius Beaker to obtain 10 g dry weight (a maximum allowance of 5 g of water will limit amount of very wet sediments) and add 50 ml of acetone.
- 5.5.3. Extract by immersing Sonifier head in sample suspension and operating for 3 minutes. Disperse the heat generated by the homogenizer by cooling the beaker in an ice water bath during the extraction. The increase and decrease in vibration intensity should be gradual to prevent damage to the Sonifier. Rinse Sonifier head with 1-2 ml acetone. Let acetone drip into sample beaker.
- 5.5.4. Allow suspension to settle before filtering.
- 5.5.5. Insert a glass fibre filter and a 2 cm layer of Celite 545 onto the sintered disc of the Buchner funnel and rinse the whole assembly with 20-30 ml acetone. Discard rinsings.
- 5.5.6. Filter sample supernatant through Buchner filter into cylindrical separatory funnel, with vacuum.
- 5.5.7. Add a further 50 ml acetone to remaining sediment and repeat extraction.

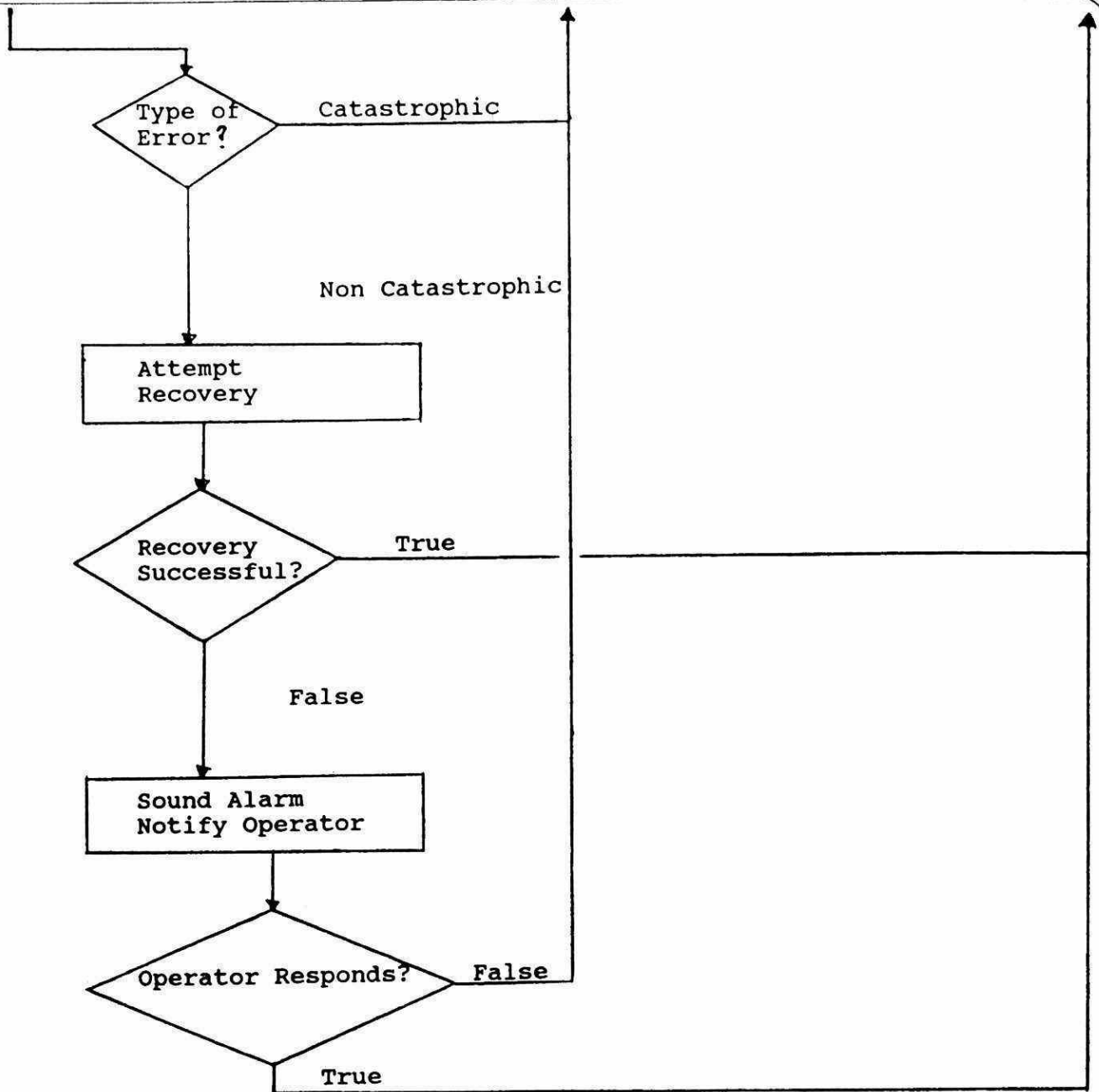
APPENDIX B

ACID DIGESTION/SOLVENT EXTRACTION FLOWCHART









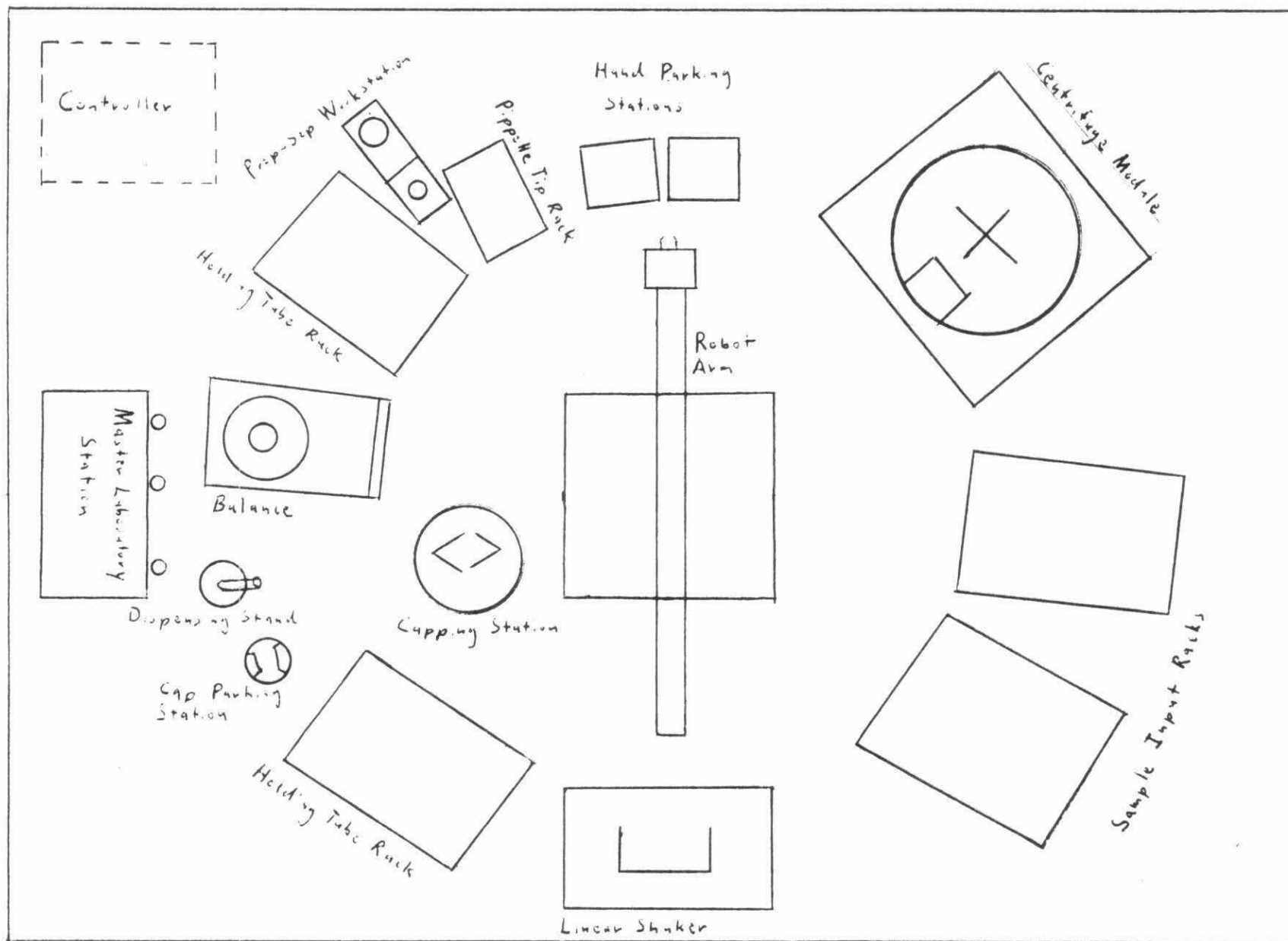


Figure I - Bench Layout

SEPARATION AND IDENTIFICATION OF ORGANIC COMPOUNDS IN
AIR PARTICULATE EXTRACTS BY HPLC AND GC/MS

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ABSTRACT

The organic compounds in air particulate extracts were separated into acidic, basic, and neutral fractions by acid/base extraction. The neutral fraction consisted of a large number of aliphatic and aromatic compounds. The identification of aromatic compounds that co-eluted with other compounds was very difficult by gas chromatography alone. Semi-preparative high pressure liquid chromatography was used to separate aliphatic compounds into one fraction and the aromatic compounds in several subsequent fractions. The retention behavior of aromatic compounds on normal phase columns in high pressure liquid chromatography was found to be typical, where capacity factors increased with an increase in the number of rings and was not largely affected by the alkyl substitution or the position of alkyl substitution on a particular aromatic compound. The high resolution gas chromatography and mass spectrometry were used for compound identification. The advantages of acid-base extraction and high pressure liquid chromatography prior to GC-MS analysis are discussed in detail.

INTRODUCTION

The identification of organic compounds in ambient air have become increasingly important because of the presence of numerous toxic compounds. Many polynuclear compounds are on the Environmental Protection Agency (EPA) priority pollutants list because they are known or suspected to be mutagenic and/or carcinogenic (1). Previously, procedures using dimethyl sulfoxide and pentane for the separation of aliphatic and polynuclears in air particulate extracts have been reported (2). The various methods for the separation and identification of polynuclear aromatics in environmental and biological samples have been reviewed several times (3-8). Present instrumental methods with conventional detectors are not able to detect the traces of toxic pollutants directly in the air. Most of the work aimed at such an analysis involves collection of airborne particulate matter by high volume filtration techniques using glass fiber filters as collection media (9,10). The extraction of organic compounds from such filters and the extraction efficiency using various solvents is reported in the literature (11,12). The identification of a large number of compounds overlapping in gas chromatographic analysis is very difficult due to interference. These

compounds can be separated applying different techniques based on their chemical properties. Acid/base extraction for the separation of compounds into acidic, basic, and neutral fractions has been reported in the literature (13). There has been a great upsurge of interest in high performance liquid chromatography(HPLC) for the separation of various compounds based on their polarities. Semi-preparative HPLC has been used for the separation and isolation of various compounds into definite compound classes. An optimized HPLC program developed using standards can be used for the analysis of real samples because of the high reproducibility of HPLC method. Because of the high capacity and low efficiency of semi-preparative HPLC the identification of traces of compounds of interest is difficult by HPLC detectors. However, the HPLC fractions after concentration can be analysed very easily by capillary gas chromatography(GC). Thus, combination of more than one techniques for analysis of complex samples results in separation and identification of large number of compounds. We will report in this paper the use of acid/base extraction, semi-preparative HPLC, and gas chromatography mass spectrometry for the complete analysis of organic compounds in air particulate extracts. Samples were obtained from the Ontario Ministry of Environment, and were collected near a wood burning facility.

EXPERIMENTAL

Chemicals and Methods: The solvents used in this method were distilled in glass, UV grade from Caledon Laboratories, Ltd. (Georgetown, Ontario, Canada). The standard polyaromatic hydrocarbons (PAH) were purchased from Ultra Scientific, Inc. (Hope, RI) and Aldrich Chemical Company, Inc. (Milwaukee, USA). All the standards were prepared by dissolving 4 to 10 milligrams of each polyaromatic hydrocarbon in to an appropriate amount of benzene or mixture of benzene and dichloro methane and stored at below 0⁰ C when not in the use.

The general scheme for the separation of organic compounds in air particulate extracts is shown in Figure 1. The air particulate extracts dissolved in toluene contained suspended particulate matter which was removed using a centrifuge. The compounds were then separated into three major groups, acidic, basic and neutral using the acid base extraction. The neutral organic compounds were further separated according to their polarities using HPLC.

Semi-preparative HPLC:

The separation and fraction isolation was achieved on a Varian 5000 Liquid Chromatograph with a

Vista 401 data system which has the capability to recalculate and replot the stored data of a particular analysis. A semi-preparative μ bondapack (10 μ m) amine column (250 mm X 9.4 mm, I.D. Waters Scientific Ltd. Mississauga, Ontario, Canada) was used. The samples were injected by an automated Rheodyne injector with 100 μ l sample loop. A gradient solvent program was developed for the separation of a standard mixture containing two to five ring PAH. The program consisted of 98 % hexane and 2 % dichloromethane for 12 minutes programmed to 100 % dichloromethane at 45 minutes, held for 10 minutes and then linearly programmed to 100 % acetonitrile held for 10 minutes and then programmed back to 100 % dichloromethane and finally to hexane. The fractions were collected between 6 to 14, 20, 24, 28, 33, 37, 43, 55 and 80 minutes. All fractions were concentrated to 200 μ l each for GC and GC/MS analysis.

GC Analysis:

The HPLC fractions were analyzed on a Hewlett Packard 5880 A GC equipped with flame ionization and electron capture detectors using a 30 m X 0.32 mm I.D. DB-5 fused silica capillary column (J and W Scientific, Inc., Rancho Cordova, CA, USA). The same column was used for the separation and characterization of individual compounds by GC/MS.

GC/MS Analysis:

GC/MS analysis was performed on a Hewlett Packard 5987 A with an HP 1000 data system which stores the raw data and permits drawing the mass spectra of any peak on the total ion current (TIC) trace. The library search system consists of probability based matching (PBM) based on 78,000 reference spectra. An ion source temperature at 200⁰C was used the GC conditions were similar to those used in GC/FID analysis.

RESULTS AND DISCUSSION

It is very difficult to completely analyse the air particulate extracts for organic compounds by GC alone because of peak overlap. The relative amounts of organic compounds in air particulate extract are varied and hence sample cleanup and concentration is necessary to remove interferences and to separate the compounds into different classes based on their properties such as acidity, basicity, polarity, volatility and structures using the different techniques prior to GC analysis. The general scheme for the separation of compounds into different classes is shown in Figure 1. The acid/base extraction is very important step

to remove the water soluble inorganic impurities, and highly polar acidic and basic organic compounds from the neutral organics. From our experiments, we observed that the composition of the neutrals did not changed for a period of the several months. The probable reason for this stability might have been due to the removal of the catalytic inorganic impurities by the acid/base extraction. Such impurities can catalytically effect the changes in composition of untreated extracts (14). Similarly, we have observed that the GC column deteriorate almost immediately and peaks lose their symmetry if a sample without acid/base extraction was injected.

The neutral fraction after acid/base extraction consists mainly of aliphatic and aromatic compounds. The aliphatic compounds were highly concentrated and co-eluted with environmentally important polyaromatic compounds in GC analysis. The aliphatic compounds were separated from the aromatics using the normal phase HPLC. The reproducible separation of aliphatic compounds from aromatics was obtained on both silica and amine columns in HPLC. The gradient solvent program was optimized for HPLC using a mixture of standard PAHs. It was observed that the amine column is more selective than the silica column for the

separation of PAH according to the number of rings. It was also observed that the best separation of aromatics having two and three rings can be obtained using nonpolar hexane for longer period of time or with higher flow rate. Prolonged application of hexane, however, results in peak broadening for the larger PAHs and they elute in more than one HPLC fraction. To overcome this difficulty the initial gradient program was modified from 100 % hexane to a composition of 98:2 hexane and dichloromethane used isocratically. Using this program it was observed that the PAH eluted faster where as the retention order remained the same. This program was used for the separation of neutral organics in various fractions.

In Figure 2, the UV trace, solvent gradient and flow gradient used for the analysis of organic neutrals by HPLC are shown. The fraction 1 (fig.3) exclusively consisted of aliphatic compounds. The majority of the GC peaks were identified by comparing the retention times of standards under identical conditions. The compounds identified are listed in Table 1. The peaks eluting after 45 minutes in Figure 3 are unsaturated aliphatic hydrocarbons identified by their mass spectra and a PBM library search.

The fraction 2 consisted of the two ring aromatics such

as naphthalenes, biphenylenes and their alkyl substituents. These compounds are relatively highly volatile and hence their isolation and concentration without loss was very difficult. The peaks identified by PBM search are listed in Table II.

Fractions 3 and 4 mainly consisted of three ring PAH. It is seen from the Figures 4 and 5 that several compounds identified (Table II) have similar retention times using GC but can very well be separated by HPLC. For example 4H-Cyclopenta (def) phenanthrene exclusively eluted in HPLC fraction 3, while C₁-substituted three ring PAH with identical retention times on GC eluted in the HPLC fraction 4. Similarly naphthalene , 2-phenyl (Peak no.4 fig. 5) eluted in the HPLC fraction 4, the co-eluted compound by GC was separated only by HPLC. Several other peaks were also identified in fraction 3 that are not present in fraction 4 but have identical retentions on GC. The HPLC fractionation, therefore, resulted in the separation of a large number of compounds that would otherwise have identical retentions in GC analysis.

Fraction 5 consisted mainly of 4 ring PAH and is shown in Figure 6. All the peaks were identified by PBM search while fluranthene, pyrene, benzo(a)anthracene , triphenylene were confirmed by injecting the standard under identical conditions.

The retention behaviour of polar compounds is different than the aromatic hydrocarbons on the amine column in HPLC. Even though the three ring PAH eluted in fraction 3, the three ring aromatics containing a carbonyl group were eluted in fraction 6 (fig.7). This indicated that amine column has a high selectivity to separate aromatic hydrocarbons according to the number of rings, whereas the carbonyl substituted aromatics were retained very strongly. Fractions 7-9 do not show any peaks on GC/FID.

The highly polar and reactive (acidic) compounds isolated in the acid/base extraction and identified by GC/MS using the PBM search are reported in Table III (fig. 8). The acid/base extraction have two major advantages, one is to remove the inorganic impurities and the water soluble compounds that may be permanently retained on the HPLC column. The second is to separate the polar reactive organic compounds which otherwise require the application of very strong solvents and high flow rate for their elution from the HPLC amine column. The recoveries for selected PAH after acid base extraction were above 85 %. This indicates the simplicity and usefulness of an acid/base extraction prior to HPLC and GC/MS analysis.

CONCLUSION

A normal phase semi-preparative HPLC method using an amine column gave complete separation of aliphatics from aromatics and aromatics according to the number of rings. The complete analysis of environmental complex samples can be carried out using an acid/base extraction followed by HPLC and GC/MS analysis. This technique is very useful when the sample contains a large number of compounds having varied chemical and physical properties.

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TABLE I : Organic compounds identified in the HPLC fractions of air particulate extract.

Peak No.	Compound	RT (min)	Mol Wt.
Fraction 1 (Figure 3)			
1	Heptadecane	18.12	240
2	Octadecane	20.62	254
3	Nonadecane	23.23	268
4	Eicosane	25.74	282
5	Henecosane	28.10	296
6	Docosane	30.35	310
7	Tricosane	32.11	324
8	Tetracosane	34.59	338
9	Pentacosane	36.59	352
10	Hexacosane	38.53	366
11	Heptacosane	40.38	380
12	Octacosane	42.11	394
13	Nonacosane	43.96	408
14	unsaturated aliphatics		
Fraction 2			
1	Naphthalene, dimethyl	11.53	156
2	Naphthalene, dimethyl	12.48	156
3	Dibenzofuron	14.63	168
4	9H-Fluorene	16.49	166
5	9H-Fluorene, methyl	17.17	180
6	Dibenzofuran, methyl	17.61	182
7	Fluorene, methyl	19.69	180
8	Benzene, 1-methyl-2-(2-phenyl-ethenyl)	22.79	194
9	9H-Fluorene, dimethyl	23.00	194
10	5H-Dibenzo(a,d)cycloheptene, 5-methylene	23.97	204
11	Naphthelene, 1-(phenylmethyl)	26.88	218
12	Benz(a)anthracene, 1,4,7,12-tetrahydro-	29.28	232

Table 1. Organic compounds identified in the HPLC fractions of air particulate extract (continued...)

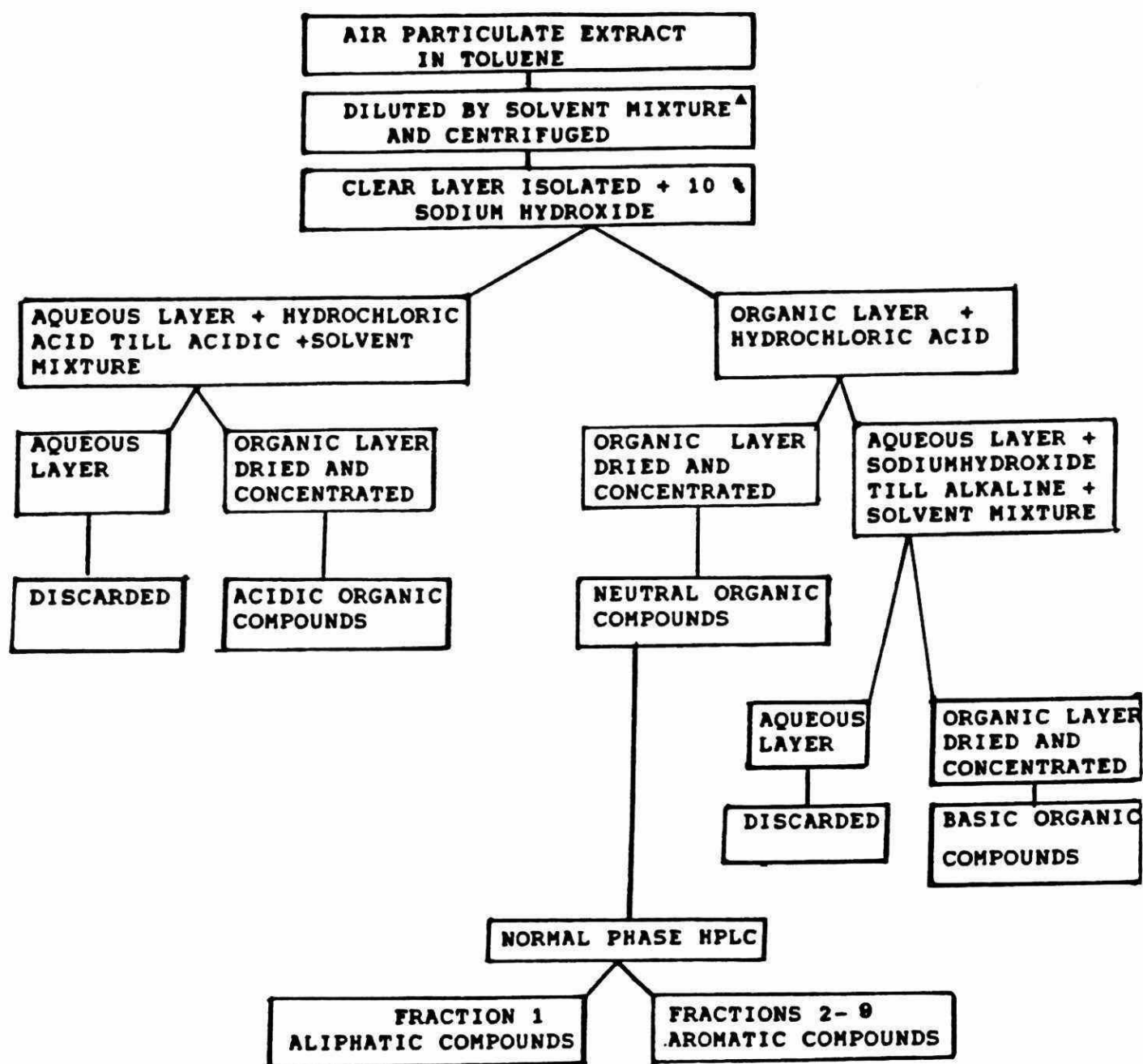
Peak no	Compound	RT (min)	Mol.Wt.
Fraction 3 (Figure 4)			
1	Phenanthrene	23.02	178
2	Anthracene	23.28	178
3(a-b)	C1-Substituted Phenanthrene/ Anthracene	24.00	192
4	1H-Indene,1-(phenylmethylene)	25.00	204
5(a-c)	C1-Substituted Phenanthrene/ Anthracene		192
6	4H-Cyclopenta(def)phenanthrene	24.46	190
7	C1-Substituted Phenanthrene/ Anthracene	27.04	192
8	Benzene 1,1'-(1-Cyclobutene- 1,2-diyl)bis	28.00	206
9	Naphthylene, 2-phenyl	28.40	204
10(a-c)	C2-Substituted Phenanthrene/ Anthracene	29.85	206
11	Indenoindene	31.53	204
12	Pyrene,4,5-dihydro	31.96	204
13	Naphthalene,2 (phenylmethyl)	32.36	218
14	1,4-Naphthalenedion,5,8-di- methoxy	32.83	218
15	C3-Substituted Phenanthrene/ Anthracene	34.16	220
16(a-d)	Azulene,2,6-dimethyl-4-phenyl	36.39	332
Fraction 4 (Figure 5)			
1	Phenanthrene	23.06	178
2	Anthracene	23.30	178
3(a-d)	C1-Substituted Phenanthrene/ Anthracene	26.7	192
4	Naphthylene,2-phenyl	28.03	204
5(a-e)	C2-Substituted Phenanthrene/ Anthracene	28.98	206
6	Pyrene	30.96	202
7(a-c)	1,4-Naphthalenedion,5,8- dimethoxy	31.53	218
8	Disulphide,diphenyl	32.83	218
9	C1-Substituted Pyrene	34.16	216
10	Azulene,2,6-dimethyl 4-phenyl	34.51	232

Table 1. Organic compounds identified in the HPLC fractions of air particulate extract (continued...)

Peak no	Compound	RT (min)	Mol.Wt.
Fraction 5 (Figure 6)			
1	Fluoranthene	30.20	202
2	Aceaphenanthrene	30.75	202
3	Pyrene	31.19	202
4(a-e)	C1-Substituted Pyrene/11H-benzo(b)fluorene	33.05	216
5(a,b)	Chrysene/Triphenylene	35.05	228
6(a,b)	Benzo(ghi)fluoranthene	37.47	226
7(a-b)	Benzo(a)anthracene/chrysene/Triphenylene	38.74	228
8(a-c)	C1-Substituted Chrysene/Benz(a)anthracene/Triphenylene	41.34	242
Fraction 6 (Figure 7)			
1	Furan, 2-butyltetrahydro	9.7	128
2	9H-Fluoren-9-one	22.17	180
3	1-Phenanthrenol	24.6	194
4	9(10H)-Anthracenone	26.2	194
5	9,10-Anthracenodion	28.3	208
6	Phenindione	31.6	222
7	7H-Benz(de)anthracene-7-one	36.8	230
8	Benzo(ghi)fluoranthene	37.69	226
9(a,b)	Chrycene/Triphenylene/Benz(a)anthracene	38.51	228
10(a-d)	Benzofluoranthene/Benzo(a)-pyrene/Benzo(b)pyrene/Perylene	44.92	252
11	Benz(j)aceanthrylene, 3-methyl	46.80	252
12	1,2:3,4-Dibenzoanthracene	48.94	278
13(a-d)	1,2-Benzoperylene/Benzo(ghi)-perylene/Dibenzo(def,mono)-chrysene	51.81	276
14	Dibenzo(A,K)pyrene	57.84	302
15	Coronene	58.16	300
16	unknown	59.19	300

TABLE 2 : Acidic compounds identified in air prticulate extract (Figure 8).

Peak No.	Compound	RT	Mol Wt.
1	Phenol, 4-ethyl-2methoxy	6.78	152
2	Phenol, 2,6-dimethoxy	8.88	154
3	Benzoic acid, 4-hydroxy-3-methoxy-	11.36	168
4	2-Naphthalenol	12.98	144
5	Ethanone, 1-(2,6-dihydroxy-4-methoxyphenyl)-	13.76	182
6	1-Naphthalenol, 4-methyl	16.06	158
7	(1,1'-Biphenyl)-3-ol	18.66	170
8	Unknown	22.68	182
9	Methanone, (2-hydroxyphenyl)phenyl-	25.50	198
10	Phenanthrenol	28.96	194
11	Phenol, 2,6-dichloro-4-(1-methylpropyl)-	34.42	



[^] 20% DICHLOROMETHANE IN BENZENE

Figure 1. General scheme for the separation of organic compounds in air particulate extract.

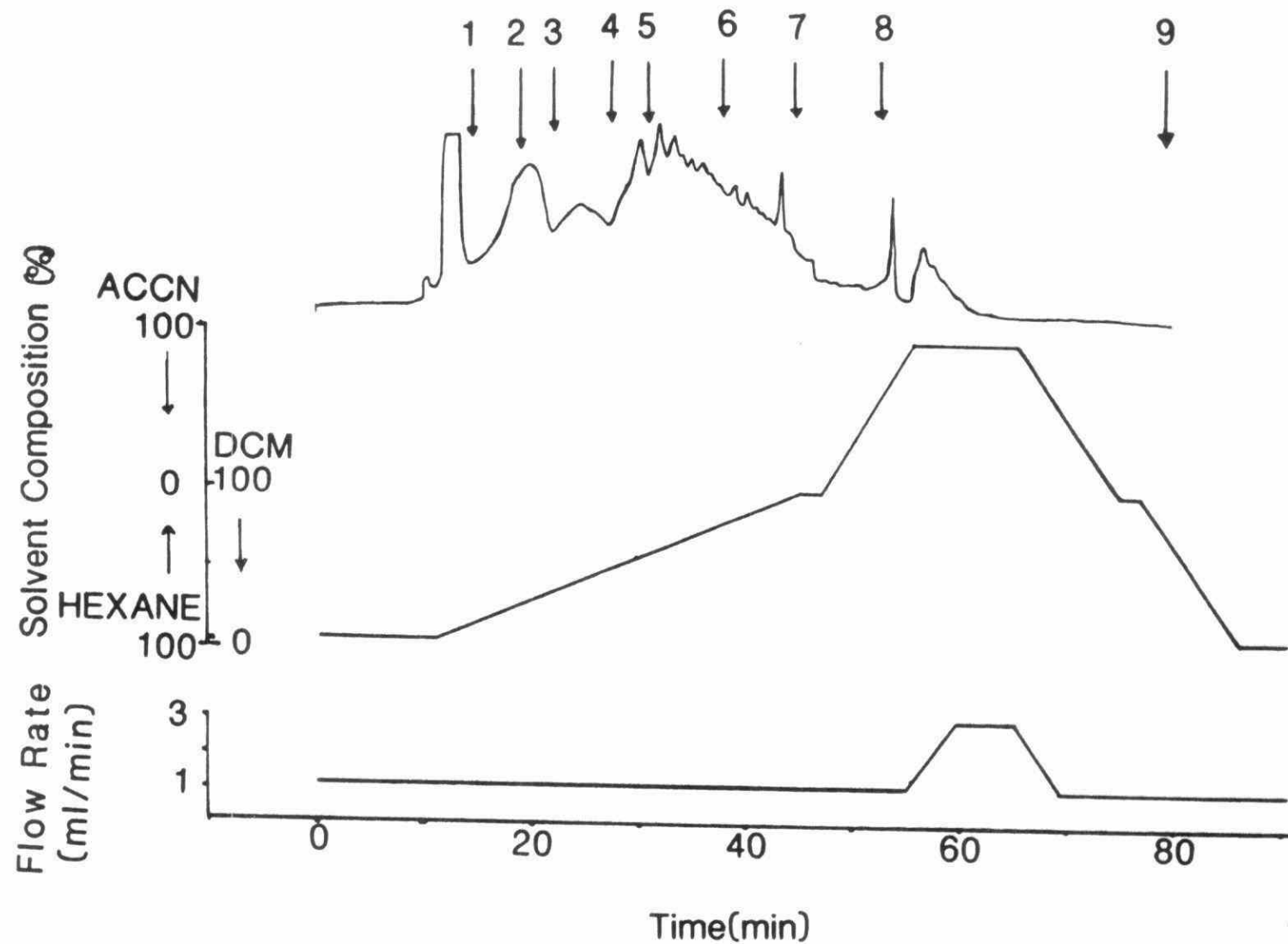


Figure 2. Scheme of the HPLC separation of neutral fraction of organics. a. UV trace in HPLC analysis, b. solvent gradient, and 3. flow gradient. Other conditions described in experimental section.

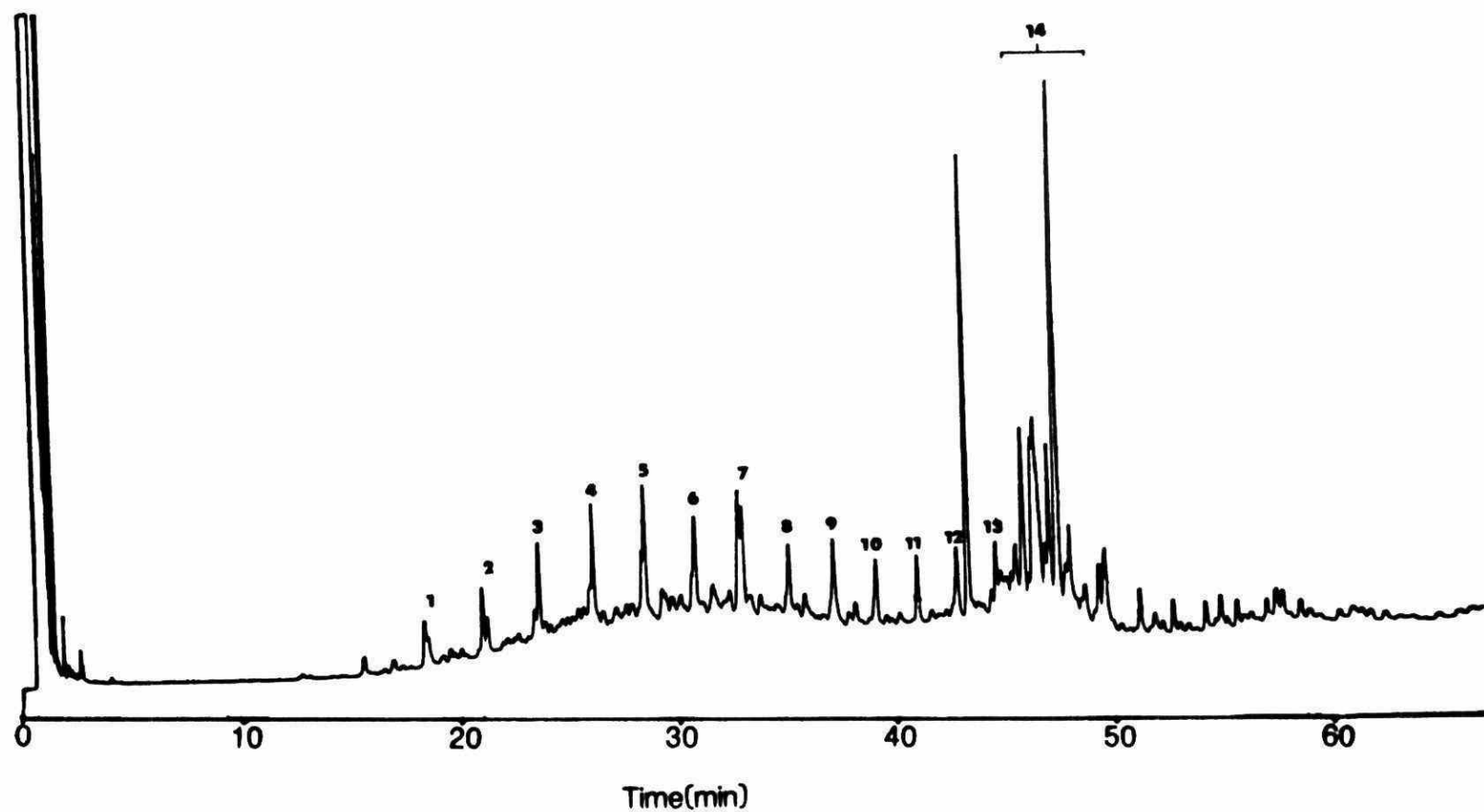


Figure 3. Gas chromatogram of HPLC fraction 1 of air particulate extract. Chromatographic conditions: 30 m X 0.32 mm I.D., DB-5 fused silica capillary column; temperature, 80 °C for 1 min, programmed to 300 °C at 4 °C/min, 20 min at 300 °C, detector FID, peaks in Table 1.

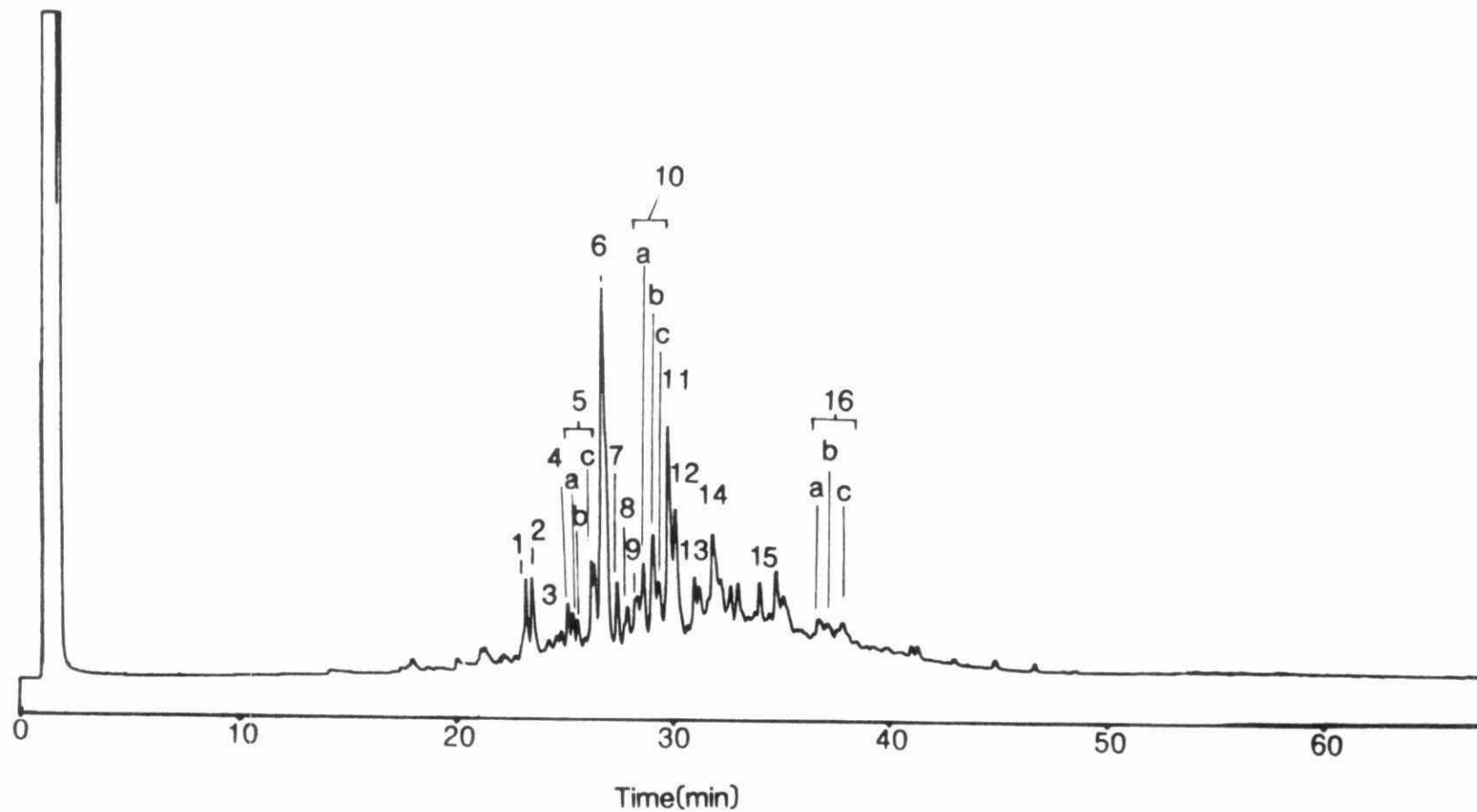


Figure 4. Gas chromatogram of HPLC fraction 3. Chromatographic conditions as in Figure 3. Peaks in Table 1.

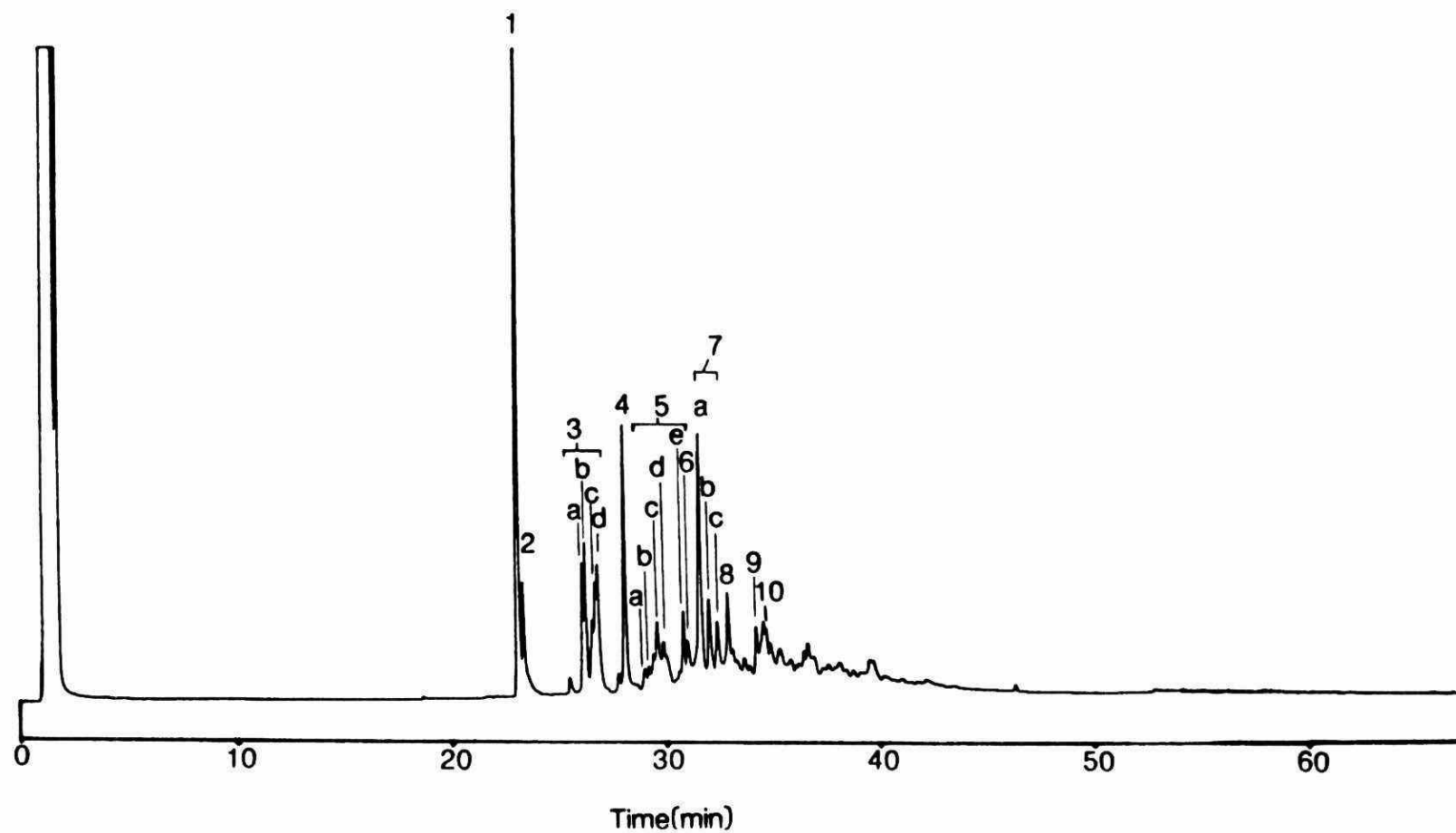


Figure 5. Gas chromatogram of HPLC fraction 4. Chromatographic conditions as in Figure 3. Peaks in Table 1.

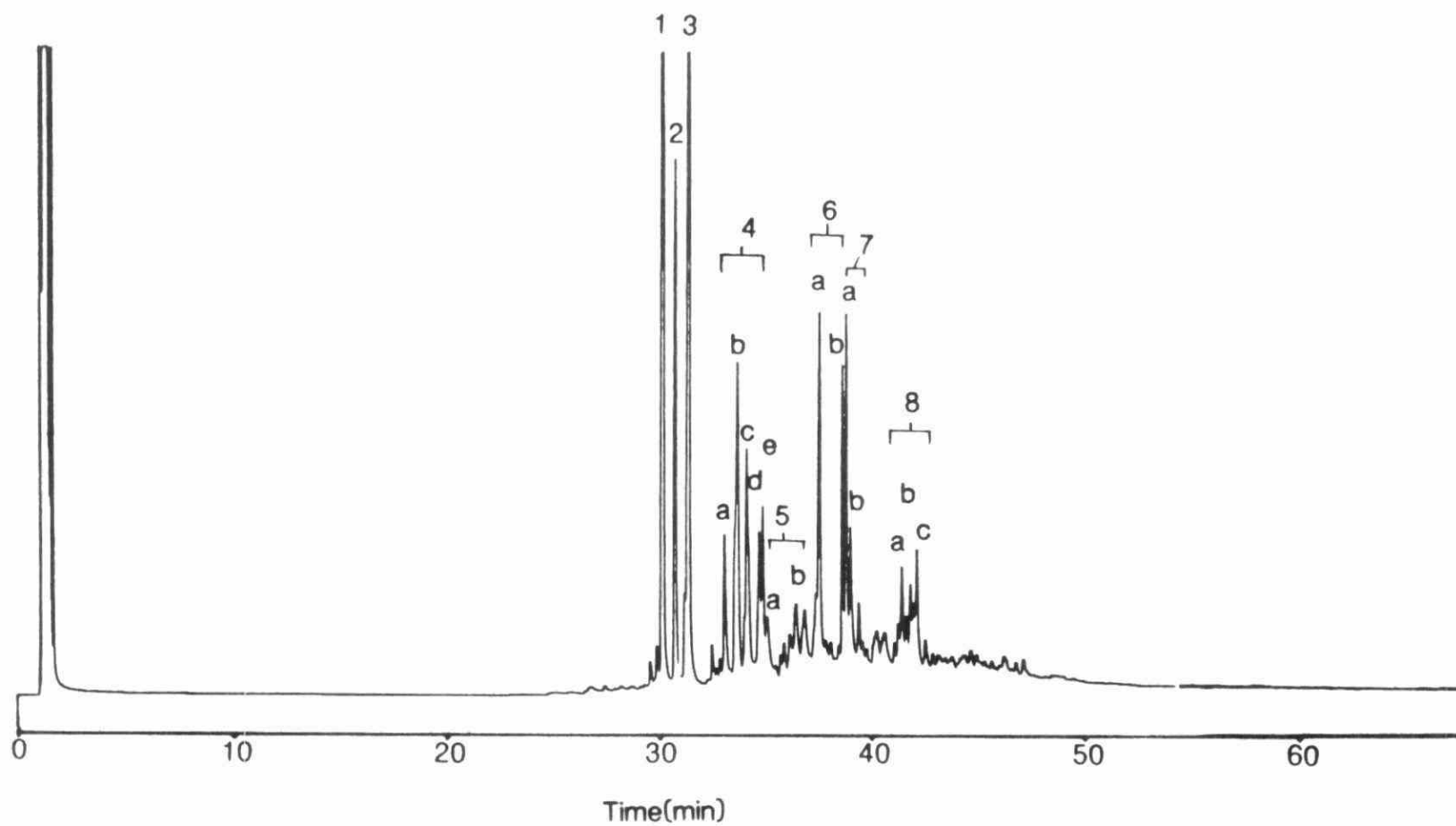


Figure 6. Gas chromatogram of HPLC fraction 5. Chromatographic conditions as in Figure 3. Peaks in Table 1.

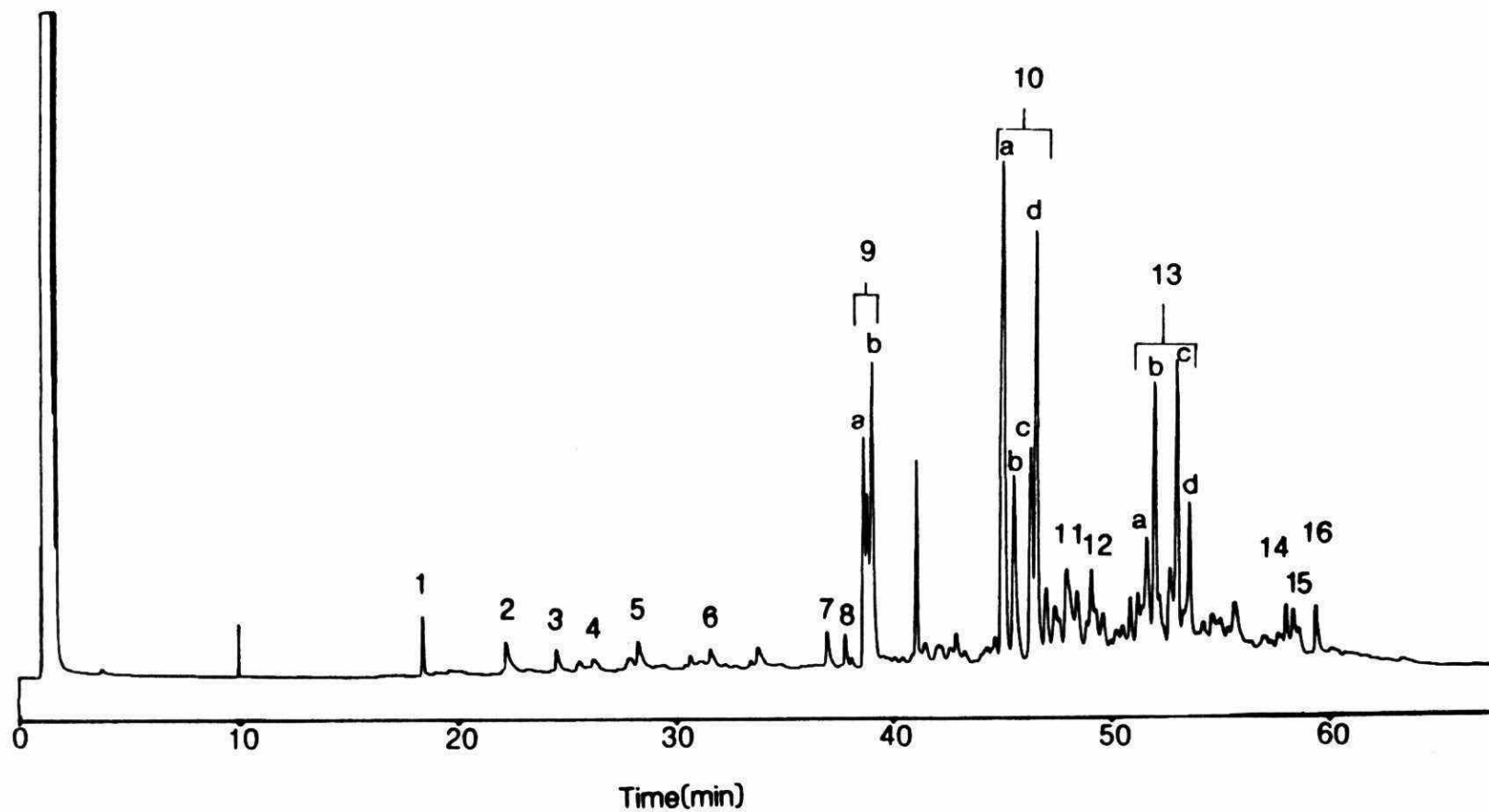


Figure 7. Gas chromatogram of HPLC fraction 6. Chromatographic conditions as in Figure 3. Peaks in Table 1.

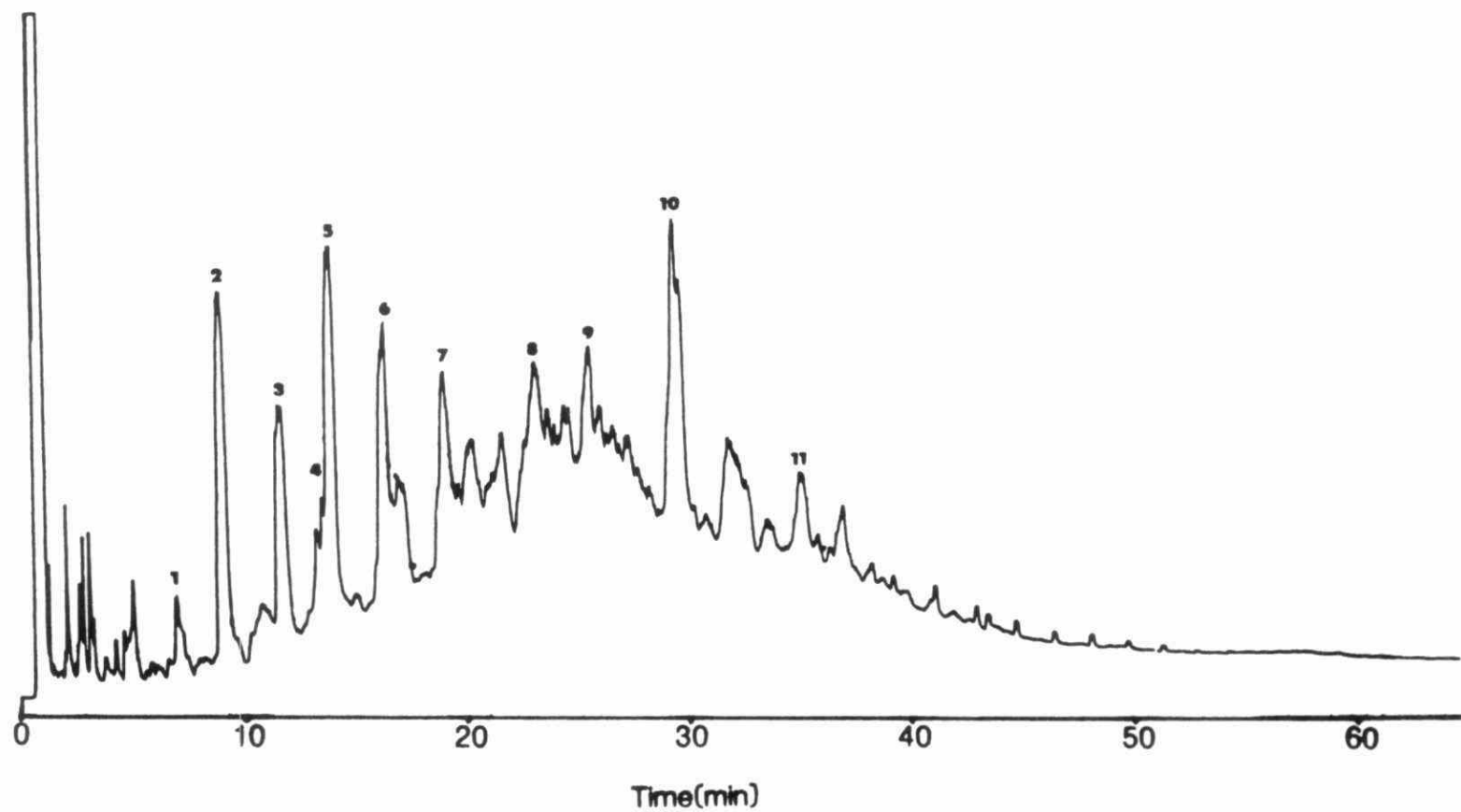


Figure 8. Gas chromatogram of acidic compounds separated by acid/base extraction. Gas chromatographic conditions as in Figure 3. Peaks in Table 2.

QUANTITATIVE DETERMINATION OF SELECTED PAH'S BY SHPOL'SKII AND JET-COOLED SPECTROSCOPY

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1. Introduction

The case has been made over the last several years for the presence of trace quantities of PAH's in the environment, some of which have been demonstrated to be mammalian carcinogens and/or mutagens. Some problems, however, remain in current analytic techniques. Certain PAH's that are physical isomers of each other cannot be resolved by liquid chromatography. This becomes of importance when one or more of the isomers are carcinogenic. Optical spectroscopy techniques are, in principle, capable of differentiating between such isomers, since structural differences between isomers can lead to substantially different spectra. An example is provided by the laser excitation spectra of benzo(a)pyrene (carcinogenic) and benzo(e)pyrene (non-carcinogenic) in cold n-octane matrices obtained in our laboratory and seen in Figure 1. The use of tunable dye lasers as spectroscopic sources has other advantages besides the type of selectivity illustrated above. The high intrinsic intensity of a pulsed dye laser makes possible detection techniques of high sensitivity. In addition, the narrow laser linewidth (typically < 0.1 nm.) allows one to separate closely spaced spectral features, and thus to minimize spectral interference effects arising from other species occurring in the sample being analysed. Further discrimination may be derived by taking advantage of varying radiative lifetimes of the component species in an environmental sample. For example, while perylene and benzo(a)pyrene, B(a)P, may potentially interfere with each other, this possibility

can be reduced or eliminated by delaying observation of the laser induced fluorescence until, say 150 ns. after the laser pulse. Since B(a)P has a radiative lifetime of 270 ns. while perylene has a lifetime of 9 ns., it is easily seen that at a delay time of 150 ns. B(a)P fluorescence will still be strong, whereas perylene fluorescence will have become undetectable.

There are, however, severe problems associated with the spectroscopy of large molecules which must be overcome before spectroscopic techniques can be used to full advantage. The fluorescence and absorption spectra of large molecules consist generally of single broad, featureless peaks extending over 10 nm. or more. This is true whether the PAH is in the form of a solid or vapour, or dissolved in a suitable solvent. It is the high number of internal degrees of freedom coupled with the presence of many vibrational modes with small characteristic frequencies that is largely responsible for this state of affairs. The low values of PAH rotational constants exacerbates the problem. Even at room temperature many vibrational modes are excited, some possessing several quanta of energy, and rotational energy levels of high rotational quantum number are also prominent. Thus, even at room temperature the PAH molecule is characterised by a high density of occupied states and this must inevitably lead to a highly congested spectrum in which individual rotational lines cannot be resolved. Under certain conditions this problem can be overcome by cooling the PAH to very low temperatures, sufficiently low that only the lowest vibrational and rotational levels will be significantly populated. In our laboratory we use two methods, which will be described below.

One method makes use of a cryogenic refrigerator to cool the PAH and is the basis for the technique known as Shpol'skii spectroscopy by which, as will be explained below, it is possible to obtain discrete looking spectra of PAH's (1). However, merely cooling the solid PAH will not produce the desired simplification

of the PAH spectrum. The PAH must first be dissolved in a suitable solvent and highly diluted. We normally use a heavy normal alkane, such as n-octane or n-hexane, as a solvent and dilute the solution to concentrations of 100 ng/mL or less. A small cell is filled with an aliquot, carefully dipped into liquid nitrogen for flash freezing and inserted into the refrigerator for further cooling to 20 K or less. The PAH molecule finding itself locked into a rigid matrix is now incapable of rotation, whereas the low ambient temperature ensures that only the lowest vibrational levels of the ground electronic state will be significantly occupied. The absorption of a resonant photon of appropriate energy will raise the molecule to an excited electronic state, which depending on the photon energy may also exhibit vibrational excitation. This excited electronic state may be either the first excited singlet or some higher state. Thus, as one scans the frequency of a laser through the absorption region of a specific PAH one will observe a number of sharp absorption peaks which measure the energy separations between the lowest lying vibrational states of the ground electronic state of the molecule and excited vibronic states. Normally, when using a laser as described above, one measures the fluorescence resulting from photon absorption by using either a monochromator set at a fixed wavelength or a suitable narrow bandpass filter rather than measuring laser beam attenuation. The resulting spectrum is called an excitation spectrum. The spectra shown in Figure 1 are examples of excitation spectra. The widths of the absorption features or quasi-lines of a given PAH are dependent on the temperature of the matrix, the nature of the solvent, the concentration of the solute and the technique used to first freeze and then further cool the sample. The linewidths decrease very rapidly with temperature to about 20K and then relatively slowly beyond 20K. At these low temperatures, widths of a few hundredths of a nanometre are typical. The solvent must be chosen carefully. Long chain normal alkanes are well suited to PAH's, because in

their frozen matrices the PAH molecules are well aligned along the lengths of the solvent molecules and this minimizes without necessarily eliminating orientation effects. Concentrations must be chosen low enough to minimize solute-solute interactions. Flash freezing the solution in liquid nitrogen forms myriad micro-crystals and reduces the chances of forming a glass, which would produce relatively broad absorption features. Finally, not all solutes are capable of showing the Shpol'skii effect.

Another type of spectrum can also be obtained from matrix cooled PAH's by electronically exciting the PAH with a laser operated at a fixed resonant frequency and dispersing the resulting fluorescence with a scanned monochromator. Such a spectrum is called a fluorescence spectrum. Depending on the energy of the absorbed photon the electronically excited PAH may also be vibrationally excited. However, non-radiative relaxation will deactivate vibrationally excited molecules in the first excited singlet state to the lowest vibrational level of the state within the radiative lifetime of the excited electronic state. Molecules excited to higher electronic states are also removed to the lowest vibrational level of the first excited singlet state with like efficiency. One consequence of this is that fluorescence spectra are independent of the excitation wavelength. Another is that the quasi-line features of fluorescence spectra measure the energy separations of vibrational levels in the ground electronic state.

The second type of cooling used in our laboratory involves a supersonic jet. The PAH is maintained at a selected vapour pressure in the stagnation chamber by heating, an inert gas is admitted to the chamber and the mixture is expanded adiabatically through a pinhole into another chamber kept at a low background pressure by fast pumping. The pinhole is chosen large enough to exceed the limits for molecular flow, but small enough not to place inordinate

demands on the pumping system. An overexpanded jet is formed in which the random molecular translational motion characterising the gas in the stagnation chamber is converted almost wholly into directed bulk flow. The translational temperature is thus reduced considerably and at 50 nozzle diameters downstream of the pinhole the temperature of a gas such as helium or argon would be less than 1K. Vibrational and rotational modes are being cooled at the same time, but with reduced efficiency. Because of the low vibrational and rotational constants of PAH's, these internal modes are also considered to be at low temperatures, which are perhaps higher than the local translational temperature, in a supersonic beam. Such a conclusion arises from the appearance of spectra obtained of beam cooled PAH's. Generally it is not practicable to determine vibrational and rotational temperatures of PAH's in supersonic beams. We have, however, measured a rotational temperature of about 6K for a NO_2 seeded argon beam at a distance of 50 nozzle diameters downstream of the pinhole. We are confident, therefore, that only the lowest rotational levels of the lowest vibrational levels of the ground electronic state of a given PAH molecule occur to any significant extent. If the laser linewidth exceeds the rotational spacing of a PAH, then the features in an excitation spectrum will represent individual vibrational bands of the electronic transition being probed and the widths of these bands will reflect the convolution of the rotational band profile and the laser line shape. In excitation spectra the observed bands measure the energy separations of individual vibrational levels of the excited electronic state and those populated in the ground electronic state. It is also possible to obtain fluorescence spectra of PAH's in cooled beams, but our experiments did not include such measurements and consequently such fluorescence spectra will not be discussed in this report.

There are similarities as well as differences between jet-cooled and Shpol'skii spectra. Since PAH molecules are essentially isolated and in a collision

free environment, internal conversion is not an important process in supersonic beams. It is, however, of crucial importance in a frozen matrix. Consequently fluorescent spectra of PAH's in a jet beam are expected to be richer than in a frozen matrix since these are expected to show transitions from excited vibrational levels of the excited electronic state. In the frozen matrix the PAH-solvent interactions shift the energy levels of the PAH molecule whereas intermolecular interactions in the jet beam are essentially negligible. As a result, a given Shpol'skii feature occurs at longer wavelengths than the corresponding cooled beam feature. Another related feature in Shpol'skii spectra is the occurrence of multiplet structures arising from different orientations of the PAH within the matrix, an effect absent in cooled jet spectra. The width of a Shpol'skii quasi-line feature is determined mainly by photon interactions with the PAH whereas the width of a feature in a jet cooled spectrum of a PAH is determined by the rotational population distribution at the local rotational temperature.

We have previously reported our success in using the Shpol'skii technique to identify a number of priority PAH's in an environmental sample to the Sixth Technology Transfer Conference held in 1985. Our identifications were confirmed by HPLC analysis. In the present stage of the project we are extending our investigations to develop quantitative methods based on both the Shpol'skii and jet beam techniques. Although considerable progress has been made the work has not yet been completed and what will be presented here will be in the nature of a progress report which emphasises our work in cooled beams not reported previously.

2.0 Experimental Section

2.1 Jet-Cooled Spectroscopy

The beam chamber consists of a cubic stainless steel box measuring about 30 cm along one side and fitted with aluminium flanges. The laser beam is brought into the chamber and allowed to exit from the chamber by means of 0.5 m baffle arms to the ends of which are attached quartz windows mounted at the Brewster angle. The supersonic beam nozzle is mounted with its axis pointing at a right angle to the laser beam and intersecting it. The nozzle can be moved along its axis in order to vary the downstream distance at which the laser intersects the supersonic beam. The laser beam is focussed to a spot of approximately 1 mm in diameter to reduce the level of laser radiation scattered from the beam nozzle which is only 3 mm away from the intersection point of the two beams. The laser induced fluorescence is collected by a lens, focussed onto an aperture positioned to block both scattered light from the laser and window fluorescence and finally passed through a collection of filters onto the cathode of an EMI 6256 photomultiplier. The base of this photomultiplier has been wired to give the photomultiplier a linear signal response up to an anode current of about 1 ma. The beam chamber is maintained at a background pressure of about 0.3 mTorr, while the supersonic jet is operating, by a liquid nitrogen trapped 6" CVC oil diffusion pump. A large target made of stainless steel sheet material is positioned about 10 cm downstream of the nozzle orifice to trap any PAH's in the supersonic beam impinging on it. The collected material is washed into a storage bottle and disposed of in suitable fashion.

The optical probe pulse is provided by the oscillator stage of a Lambda-Physik FL2000 tunable dye laser, which was modified by replacing the telescope beam expander with one based on four quartz prisms. With this arrangement laser linewidths of about 0.01 nm are achievable; however, in our

experiments we use a linewidth of 0.06 nm, which is sufficient for our purposes. The dye laser is pumped by a 3 mJ pulse from a Molelectron nitrogen laser operated at a repetition rate of about 10 Hz. The laser wavelength is determined with the aid of a 1/3 m Heath monochromator. The dye laser energy is measured absolutely with a pyroelectric detector and relatively by either of two methods. In one method, a portion of the beam is removed with a microscope slide and viewed with a blue sensitive photodiode. In the second method, the monochromator is set at the wavelength of the PAH band being excited and the intensity of the laser beam at this wavelength is monitored with a RCA 1P28 photomultiplier at the exit slit. The wavelength of the laser beam is determined with the aid of a Heath 1/3m monochromator.

Initially a pulsed NRC BV-100 beam valve was used fitted with a 0.5 mm orifice; however, this valve could not be operated at the temperatures needed to generate useful vapour pressures of PAH's. Consequently a continuously operating beam nozzle was built as shown in Figure 2. Because of limitations imposed by our vacuum pumps we are required to limit the total throughput of gases into the beam chamber. The present orifice is 0.065 mm in diameter and we are obliged to keep the stagnation pressure, i.e. the total pressure of the gas on the high pressure side of the orifice, at a maximum of 200 Torr. This fortunately does not restrict the density of PAH molecules in the supersonic beam itself, since this is determined by the PAH vapour pressure maintained in the stagnation chamber and not by the total stagnation pressure. The beam nozzle is constructed of stainless steel to minimize heat conduction apart from the 4.2 cm section at the orifice end, which is constructed of copper for efficient distribution of heat. The copper end is wound with nichrome wire for the purpose of heating. The sample is placed into a small well at the end of a conical copper insert which is supported via a metal-to-glass seal by a 25 mm o.d. pyrex tube chosen to minimize heat

conduction. A vacuum seal is effected by means of an O-ring between the pyrex tube and the stainless steel body of the beam nozzle. A chromel-alumel thermocouple attached to the copper insert is used to monitor the temperature of the sample. Pre-purified argon is added to the stagnation chamber without further purification and allowed to mix with the PAH vapour prior to passage into the beam chamber via the orifice. The stagnation pressure is measured with a Matheson absolute pressure gauge and is controlled only by the gas regulator attached directly to the argon tank.

The supersonic jet would be expected to have a roughly conical shape, if it were to expand into a perfect vacuum. However, collisions between beam molecules and the ambient molecules in the beam chamber form a standing shock wave which defines the limits of the beam. This shock moreover has a concave shape as seen from the beam axis and at some distance downstream of the nozzle causes the flow lines followed by the beam molecules to converge into a shock front, called a Mach disk, which is normal to the beam axis. Throughout the region between the nozzle and the Mach disk the beam molecules continue to be cooled. However, passage of these molecules through the Mach disk results in a compression that, in fact, increases the temperature and makes the Mach disk an unsuitable region for the type of spectroscopic measurements being carried out in the present study. Under our experimental conditions (stagnation pressure = 200 Torr, background pressure in the beam chamber = 0.3 mTorr and orifice diameter = 0.065 mm) the Mach disk is calculated to occur at about 35 mm downstream of the nozzle. Our measurements are thus conducted well within the cold regions of the beam, since the supersonic beam is probed about 3 mm downstream of the nozzle. We could not confirm this directly by determining the rotational temperature of a PAH in the jet, but we did measure the rotational temperature of NO_2 seeded into an argon jet. The vibrational band obtained at 585 nm is

illustrated in Figure 3. Only a few rotational lines are seen in the P and R branches and these show a peak intensity at $J=2$ or 3. The rotational temperature is determined to be 6 K. There is no reason to believe that PAH's in an argon jet would have higher rotational temperatures. If anything, these should be lower because of the considerably smaller rotational constants of PAH's.

The preparation of samples depends greatly on the type of information being sought. In experiments conducted to obtain spectra only a quantity of solid PAH typically in the range of a mg or less is placed into the sample well of the beam nozzle insert. In experiments to determine a PAH quantitatively a method is used developed by Yamada and Winefordner (3), and it is necessary to limit the sample size to the range of 1 to 140 μg . This is achieved by first placing a precisely measured aliquot of a dilute PAH/acetone solution into the sample well using an Eppendorf micro-pipette and then driving off the solvent by heating and ventilating. The dried sample is inserted into the beam nozzle which is being maintained at the desired temperature. The sample inserted into the stagnation chamber rapidly rises to the desired temperature. The PAH vapour is mixed with an inert gas such as helium or argon and the mixture is expanded into a supersonic jet through a pinhole into a low pressure beam chamber. A dye laser is tuned to a resonant frequency and the resulting fluorescence is measured with a photomultiplier detection system - in our case the system described earlier in this section. The signal rises to some plateau value, ideally remains there briefly and then declines to zero as the sample is depleted. The signal can be displayed on a chart recorder strip chart or digitized and stored in a computer. The integral of the signal with respect to time is proportional to the amount of PAH in the sample. A calibration plot can be determined by varying the sample size. The peak height of the signal depends only on the temperature provided that there is enough material to establish equilibrium between the solid and its vapour even if

only momentarily. The duration of the signal peak is determined by both the stagnation temperature and the amount of sample. The integrated signal depends only on the amount of sample.

For this quantitative technique to be valid it is essential that the photomultiplier be operated in its linear response range and that the ground state population of the PAH not be significantly depleted by absorption of laser photons. As mentioned earlier the photomultiplier used to measure the PAH fluorescence has a linear response up to 1 ma of anode current or a 500 mv signal across a 50 ohm resistor. Care was taken to limit signal levels to below this limit by reducing the laser intensity with neutral density filters. Depletion of the ground state population of a PAH may result in a non-linearity between fluorescence signal and laser pulse energy, even when the photomultiplier is well within its linear range of operation. The range of laser pulse energies over which the fluorescence intensity shows linear behaviour varies from one PAH to another and must be set empirically for each PAH. Normally the laser pulse energy incident on the supersonic beam is varied with the aid of neutral density filters. For strong absorbers it may be necessary to use very low pulse energies. For example, it is necessary to maintain laser pulse energies at less than 0.3 μJ when studying perylene.

Analog signal pulses from the photomultiplier are processed with an Evans Associates gated integrator for noise reduction and then displayed with the aid of a strip chart recorder. The area under the resulting curve is obtained manually directly from the trace. Since this area is proportional to the laser pulse energy, the laser pulse energy is monitored and used to normalize the area. Until the present time the absolute energy has been measured immediately prior to and after a sample run and an average pulse energy determined. The two measurements have been found to agree well with each other; however, to take

into account any variations in laser energy during the course of a sample run, a separate strip chart record of the laser energy will be generated of the output of the monochromator photomultiplier and used to establish the laser wavelength.

2.2 Shpol'skii Spectroscopy

The experimental setup has been described in detail in the Proceedings of the Fifth (4) and Sixth Technology Transfer Conferences (2), and will only be given in outline here. The sample is dissolved in a normal alkane solvent, typically n-octane, and diluted to 100 ng/mL or less. An aliquot is removed with an Eppendorf micro-pipette and transferred to a cell made of copper fixed with a quartz window. This cell is inserted into a well in a copper block attached to the cold-head of a CTI-Cryogenics Model 21 refrigerator after first freezing the solution by dunking in liquid nitrogen. Spectroscopic measurements are carried out when the sample temperature reaches a value between 10 and 20 K. Both excitation and fluorescence spectra are obtained. The latter are generated with either a nitrogen laser or a dye laser tuned to a fixed wavelength that is strongly absorbed. The fluorescence from the sample is collected by a lens and focussed through a series of filters onto the slit of a 0.3 m McPherson Model 218 scanning monochromator to which is attached a EMI 6256 photomultiplier mounted in a EMI FACT-50MKIII refrigerated housing. This photomultiplier has been wired for linear signal response up to 0.7 ma anode currents. The same precautions with respect to photomultiplier saturation and ground population depletion must be exercised in these experiments as were discussed in the previous section.

In the linear range of signal dependence on laser pulse energy the amplitude of the fluorescence signal pulse coming from the photomultiplier must be dependent linearly on the concentration of PAH in the frozen matrix. This allows one to determine PAH's quantitatively. Initially one must set up a series of calibration curves for each PAH, typically in the form of signal peak intensity

plotted against laser pulse energy. However, to avoid uncertainties arising from unexpected variations in laser pulse energy or other experimental factors it is advisable to incorporate an internal standard into each sample analysed. We have selected perylene, because it is a relatively strong absorber and because its fluorescence occurs far enough from that of other PAH's in our list not to cause problems arising from spectroscopic interferences. Thus, in a typical analysis a measured amount of perylene is added to the sample to be analysed. Fluorescence is excited either by the nitrogen laser or by the nitrogen pumped dye laser tuned to a wavelength at which absorption is strong, but well to the short wavelength side of the fluorescence to be observed in order to minimize interference from scattered laser radiation. The fluorescence is dispersed by the McPherson monochromator and peaks belonging to the perylene and other PAH's in the sample are identified. Peak heights are measured and used in the quantitative determination. Because the original sample might itself contain perylene, a fluorescence spectrum of the sample is first obtained without added perylene and a second one is obtained with the added perylene. In this way we can subtract the signal associated with the perylene originally in the sample and use the residual signal as a calibration marker.

The analog signal pulse from the McPherson monochromator is processed by an Evans Associates gated integrator for signal/noise improvement and displayed on a strip chart. Peak heights are measured and taken to be relative measurements of the concentration of PAH in the sample.

3.0 Results and Discussion

3.1 Jet-Cooled Spectroscopy

Figure 3 shows a vibrational band of NO_2 associated with the absorption of laser radiation at 585.1 nm. The top trace is a laser excitation spectrum of NO_2 seeded in a supersonic jet of argon; the lower of NO_2 gas at room temperature. The jet spectrum indicates a rotational temperature of about 6 K. These spectra illustrate quite dramatically the simplification that results from cooling even tri-atomic gases. Figure 6 shows Shpol'skii and jet-cooled excitation spectra of 9-methylanthracene. The two features in the top trace obtained in the beam are the same vibrational bands as the two leftmost features in the bottom trace obtained in a frozen matrix. The jet-cooled spectrum is shifted about 1.6 nm towards shorter wavelengths with regard to the Shpol'skii spectrum. This shift arises because in the jet the PAH molecules are essentially isolated whereas in the matrix they are subject to interaction with solvent molecules.

Jet-cooled spectra of benzo(a)pyrene [B(a)P], benzo(ghi)perylene [B(ghi)P], pyrene [Py] and perylene [P] are shown in Figures 7 to 10. Each feature is actually a vibrational band with unresolved rotational structure. The half-widths of the bands shown arise mainly from the low resolution of the laser beam, which is about 0.06 nm. Fourmann et al (4) have measured the rotational band-width of perylene to be 1.8 cm^{-1} in a jet with a rotational temperature of 4 K. This corresponds to about 0.03 nm, at the excitation wavelengths used in our study.

Figure 11 consists of two plots of signal vs time obtained in a quantitative experiment on Py - one for 140 ug and the other for $1.4 \mu\text{g}$ of Py. These experiments were conducted with the gated integrator set for a signal/noise improvement ratio of about 25. We estimate that the detection limit for Py of our system as it presently stands is approximately $1 \mu\text{g}$. A pulsed supersonic beam with an open time of, say, 0.5 ms, an orifice of 0.5 mm diameter and operated at

a repetition rate of 10 Hz would reduce the detection limit by increasing the beam density and the total time required to expel the entire sample. Such a beam would only be on for 5 ms out of each second of real time.

Figure 12 plots the signal integrated over time against the mass of the sample for Py and B(a)P. Both plots were obtained under conditions where the signal was not affected by saturation effects. The high degree of linearity of the plots is evident and underlines their validity as quantitative calibration plots. We are presently determining such calibrations for P and B(ghi)P. While we have not yet attempted to analyse a mixture of PAH's let alone an environmental sample, we are confident that given our capabilities with regard to resolution, which is related to selectivity, and sensitivity we shall successfully achieve this goal. Mr. John Lai is the graduate student responsible for the beam work reported here.

3.2 Shpol'skii Spectroscopy

Our attempts to quantify this technique have been hindered by problems with the nitrogen laser dedicated to this project. Some preliminary attempts to obtain a calibration plot for B(a)P with P as an internal reference standard have demonstrated a linear dependence of signal amplitude on the concentration, but these do not represent the best that we can do. At this moment we believe that the problems with the nitrogen laser have been satisfactorily resolved and look forward to a successful resumption of our experiments.

Despite problems with the nitrogen laser progress has been made. We have now defined the limits of signal saturation and have re-measured some of the spectra under linear conditions that were reported at the previous Technology Transfer Conference. In addition we have established upper bounds to the detection limits for B(a)P and P. These are concentrations of 1 ng/mL in each case established by observing fluorescence excited by a nitrogen laser. Given that our aliquots have typically been 600 μ L delivered to a cell of 700 μ L volume, this

concentration limit corresponds to a mass limit of 0.6 pg. This is not the true mass limit, because only a small fraction of the sample is actually probed by the laser. Steps are underway to reduce the volume of the sample cell to less than 0.1 of its present value, which would then reduce our detection limit to less than a picogram. We expect to improve on this considerably by using a tunable dye laser as the excitation source, which will permit us to excite at more favourable wavelengths than the 337 nm wavelength of the nitrogen laser. This work is being conducted by Mr. Harold Malle, who is a graduate student working towards his M.Sc.

3.3 A Comparison of the Two Techniques

The most obvious contrast between jet-cooled and Shpol'skii spectroscopies lies in their sensitivities. Shpol'skii spectroscopy is by far the more sensitive of the two in quantitative determinations of PAH. This advantage may be offset by other considerations. These sensitivities were determined for pure samples of PAH. However, of greater interest are PAH's in environmental samples. The only environmental samples we have analysed so far by Shpol'skii spectroscopy were fractions from HPLC analysis, whereas we would really desire to start with the actual environmental sample itself; e.g. with the trapped material on a Hi-Vol filter. This immediately poses the problem of extraction, with possible loss of material. On the other hand, when using jet-cooled spectroscopy, we would, in principle, be able to place a section of the Hi-Vol filter directly into the heated section of the beam nozzle and in this way eliminate any extraction steps. Such a direct analysis would be able to quickly identify and quantify selected PAH's in an environmental sample and thereby play an important part in environmental analysis. For example, it could provide a quick guide to the PAH content of an environmental sample. In addition, it could serve as an indicated of how efficient various extraction and cleanup steps are that are required as

preparatory to HPLC analysis, for example, which is a common analytical tool.

In jet-cooled spectroscopy one uses up a sample during each analysis. This means that a separate sample must be analysed for each PAH chosen. The Shpol'skii technique allows one to analyse for several different PAH's using the same sample and in this way it is less cumbersome than the jet-cooled technique, when it is desired to monitor several compounds simultaneously. The two techniques are comparable when it is desired to monitor a single PAH in a number of samples.

Shpol'skii quasi-lines are less than 0.1 nm in width for matrix temperatures of 20 K or less, whereas vibrational bands in jet-cooled spectra are in the range of 0.03 nm in width. These widths determine selectivity and it is seen that the two techniques are comparable in this regard.

4.0 References

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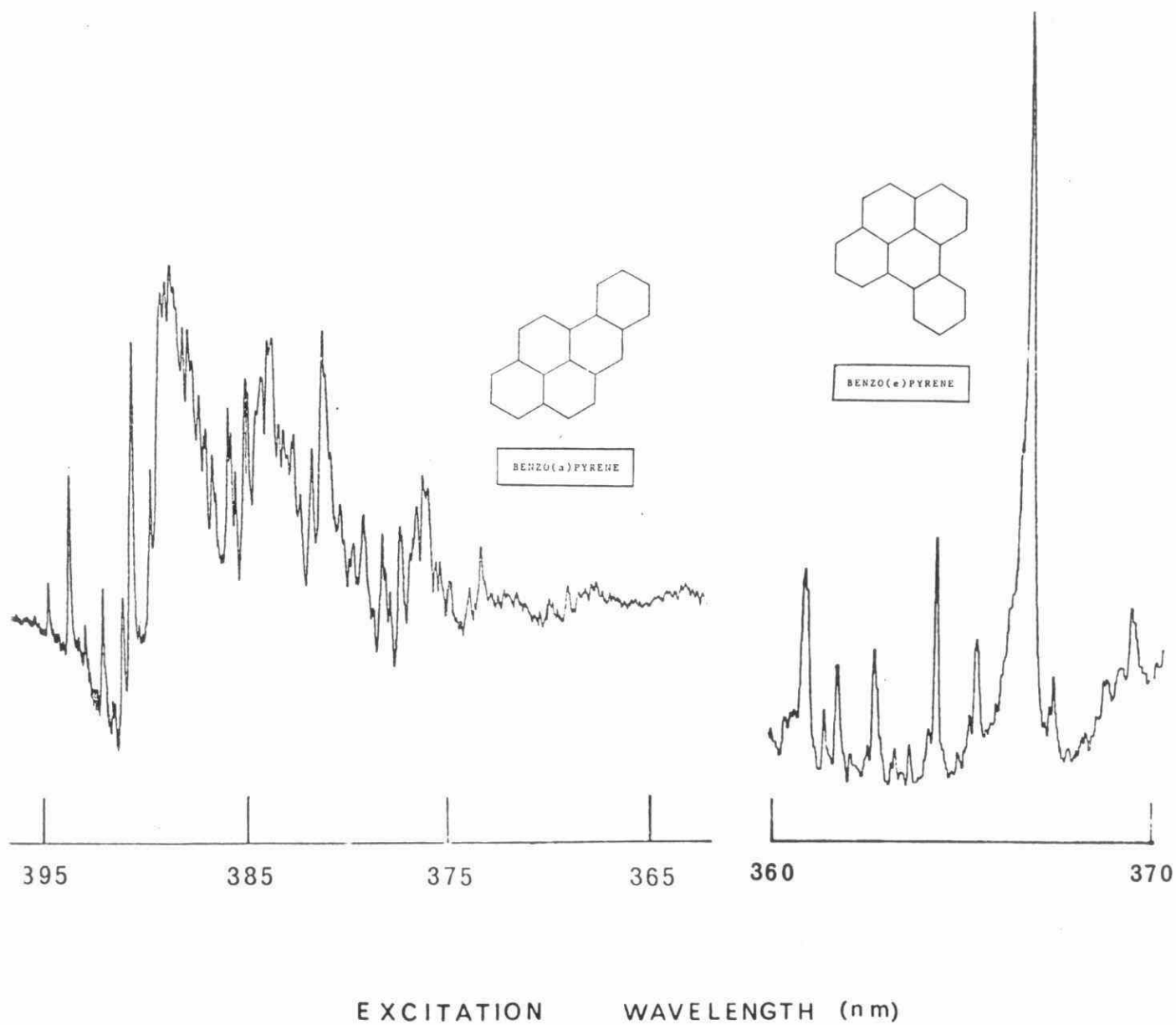


FIGURE 1: Shpol'skii Laser Excitation Spectra of Benzo(a)Pyrene and Benzo(e)Pyrene

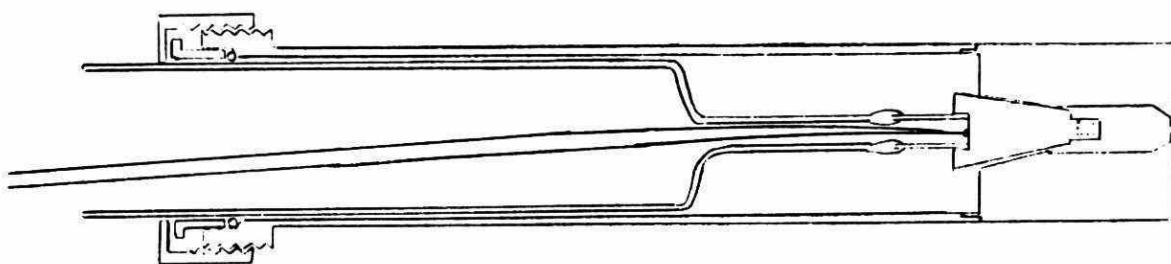


FIGURE 2: Schematic of the Supersonic Beam Nozzle

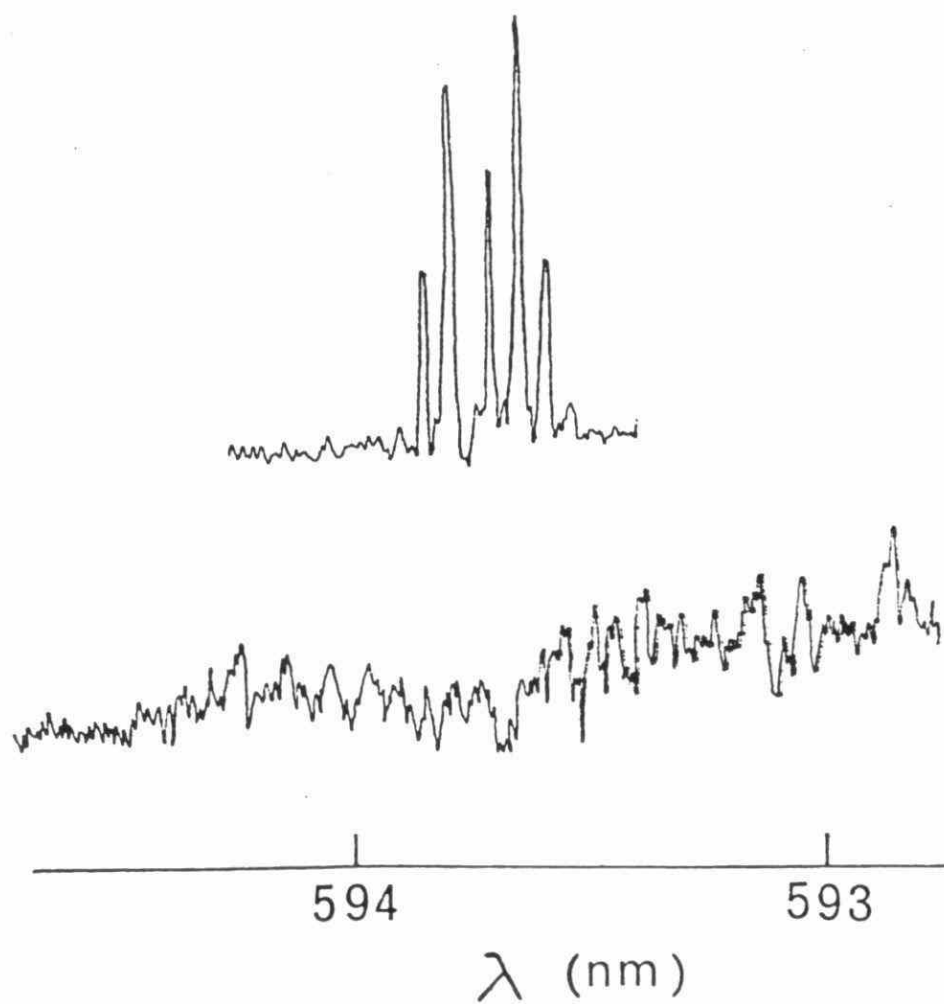


FIGURE 3: Laser Excitation Spectra of NO₂
a) Lower Trace - Room Temperature, $T = 292\text{K}$
b) Upper Trace - Jet-Cooled, $T_{\text{ROT}} = 6\text{K}$

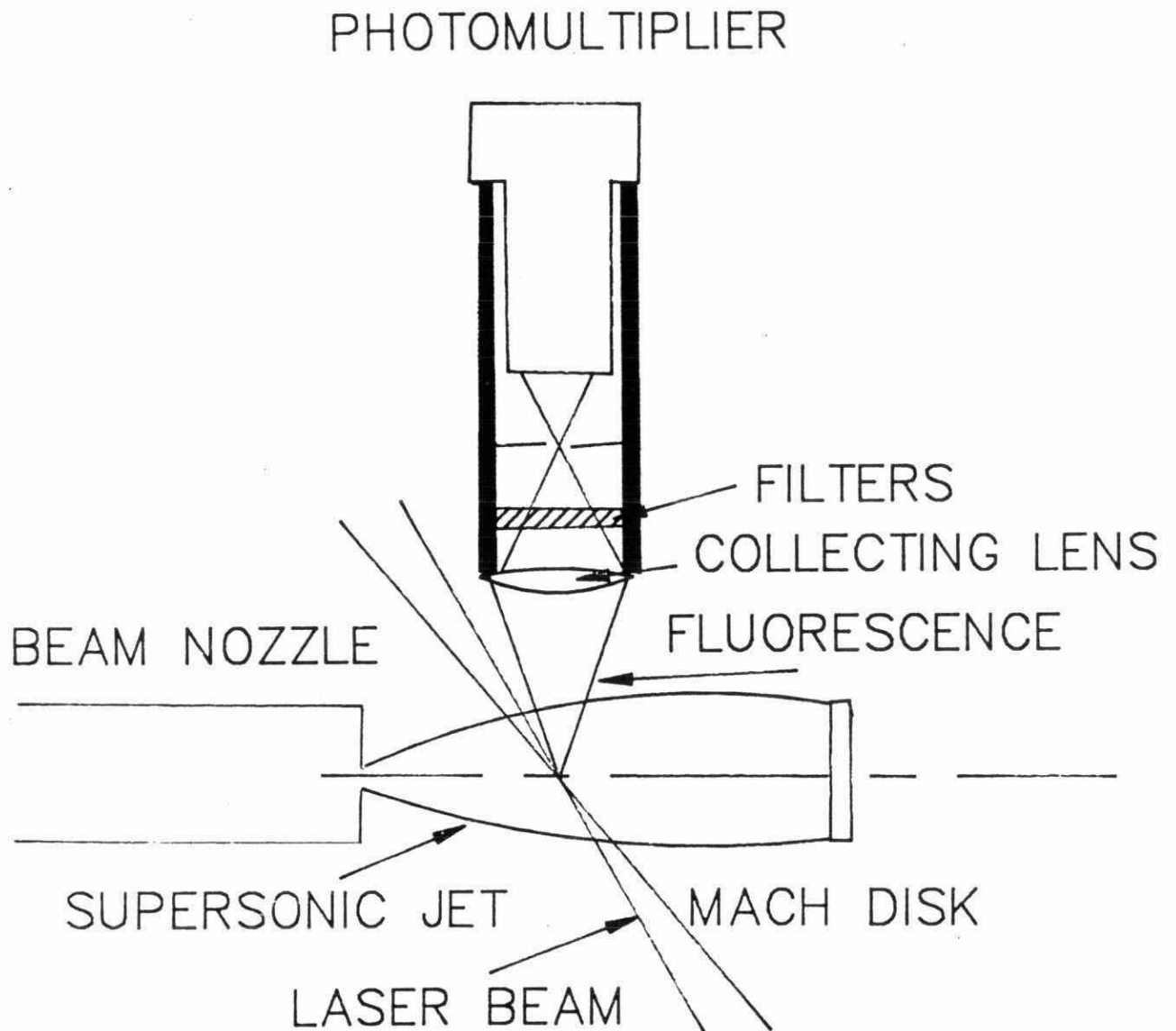


FIGURE 4: The Supersonic Beam Apparatus

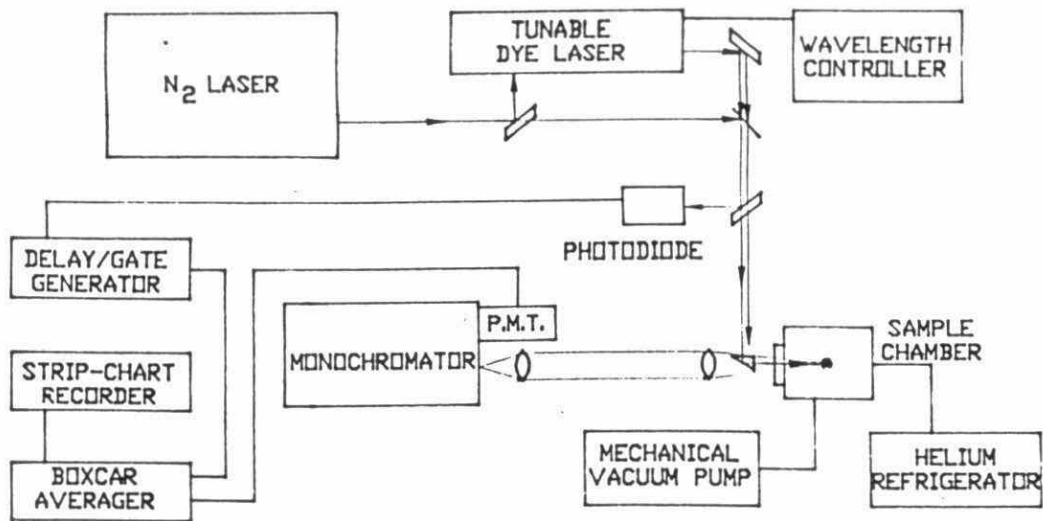


FIGURE 5: Shpol'skii Apparatus

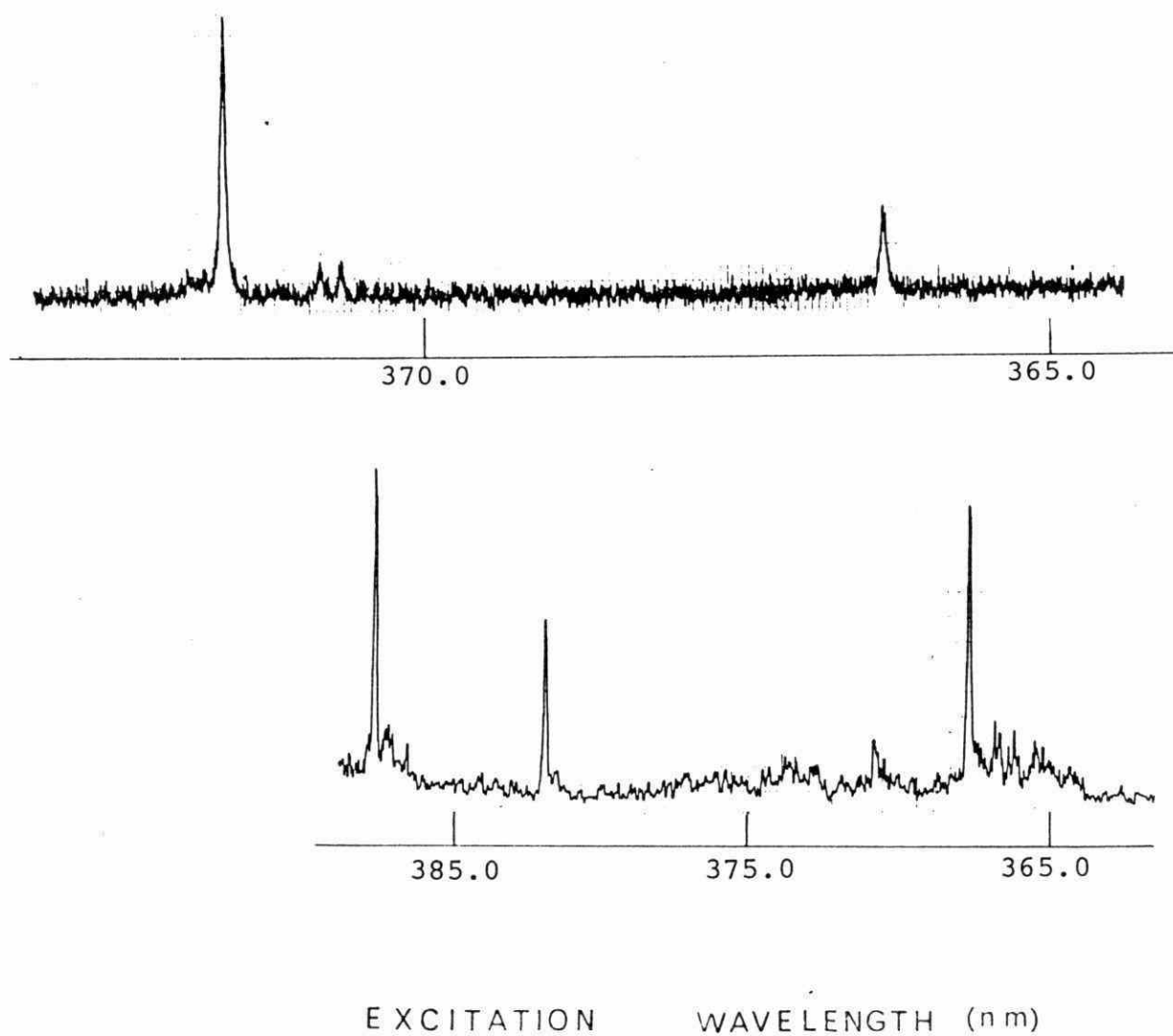


FIGURE 6: Laser Excitation Spectra of 9-Methylantracene
a) Lower Trace Matrix Cooled
b) Upper Trace Jet-Cooled

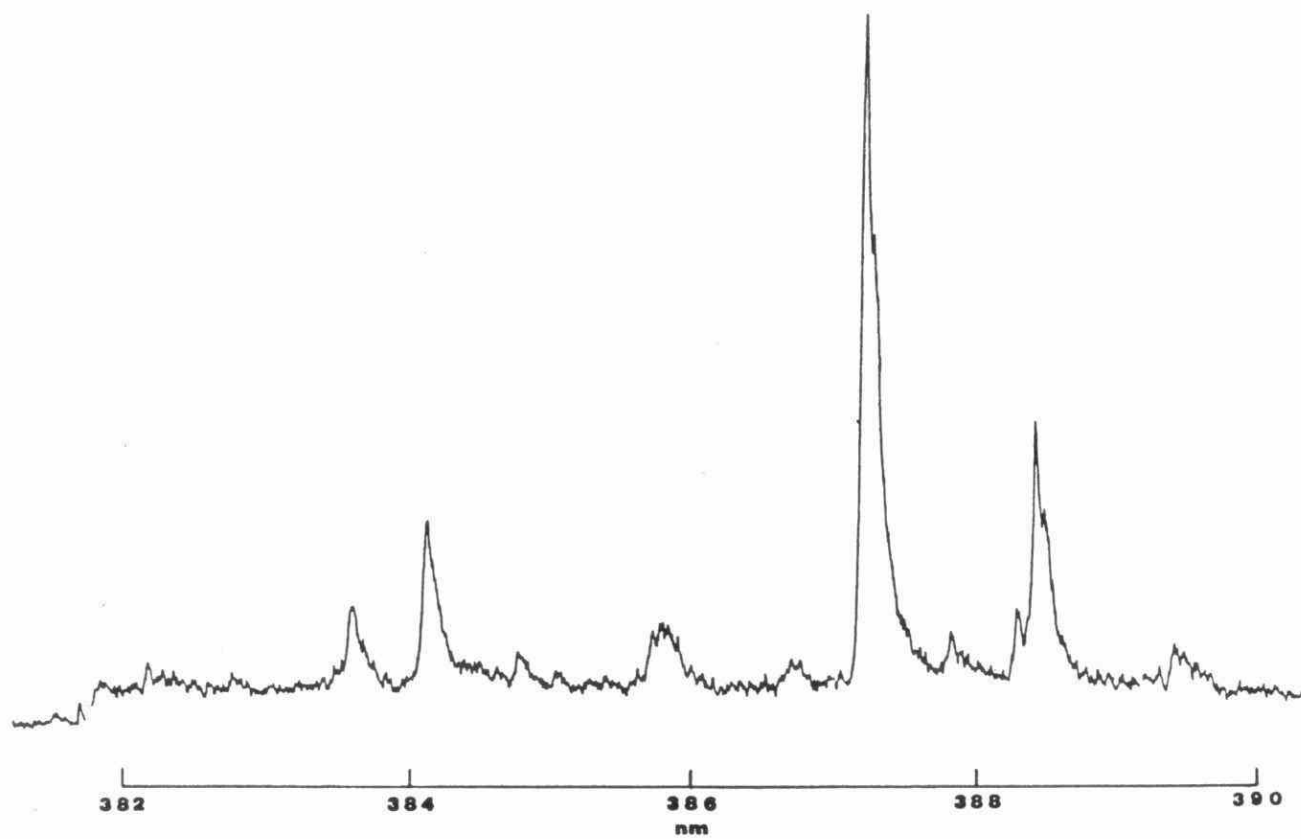


FIGURE 7: Jet-Cooled Laser Excitation Spectrum of Benzo(a)Pyrene

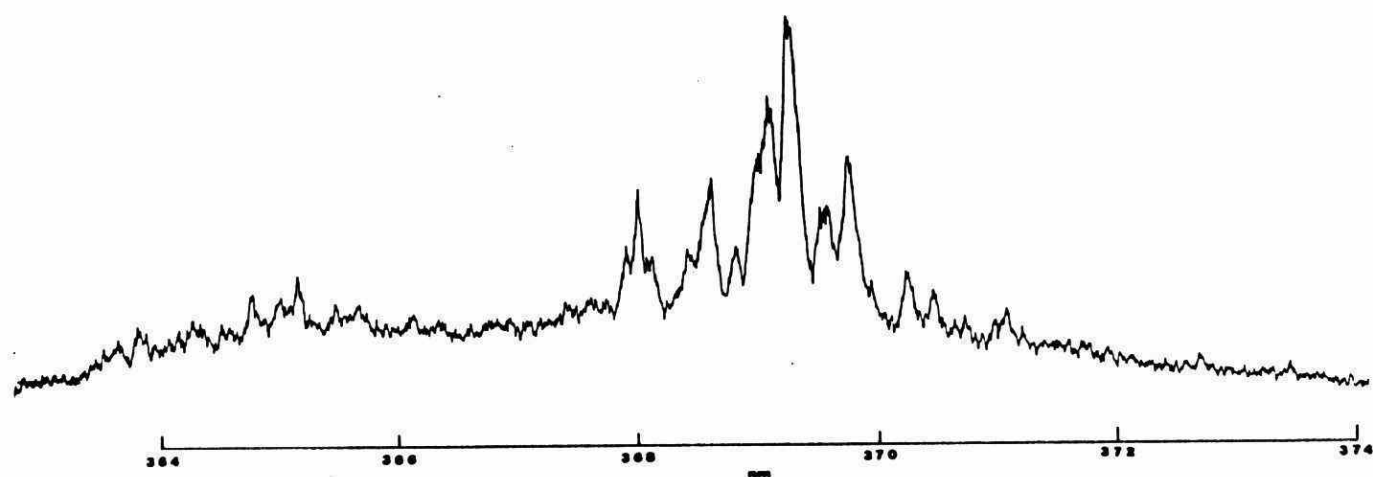


FIGURE 8: Jet-Cooled Laser Excitation Spectrum of Benzo(ghi)Pyrene

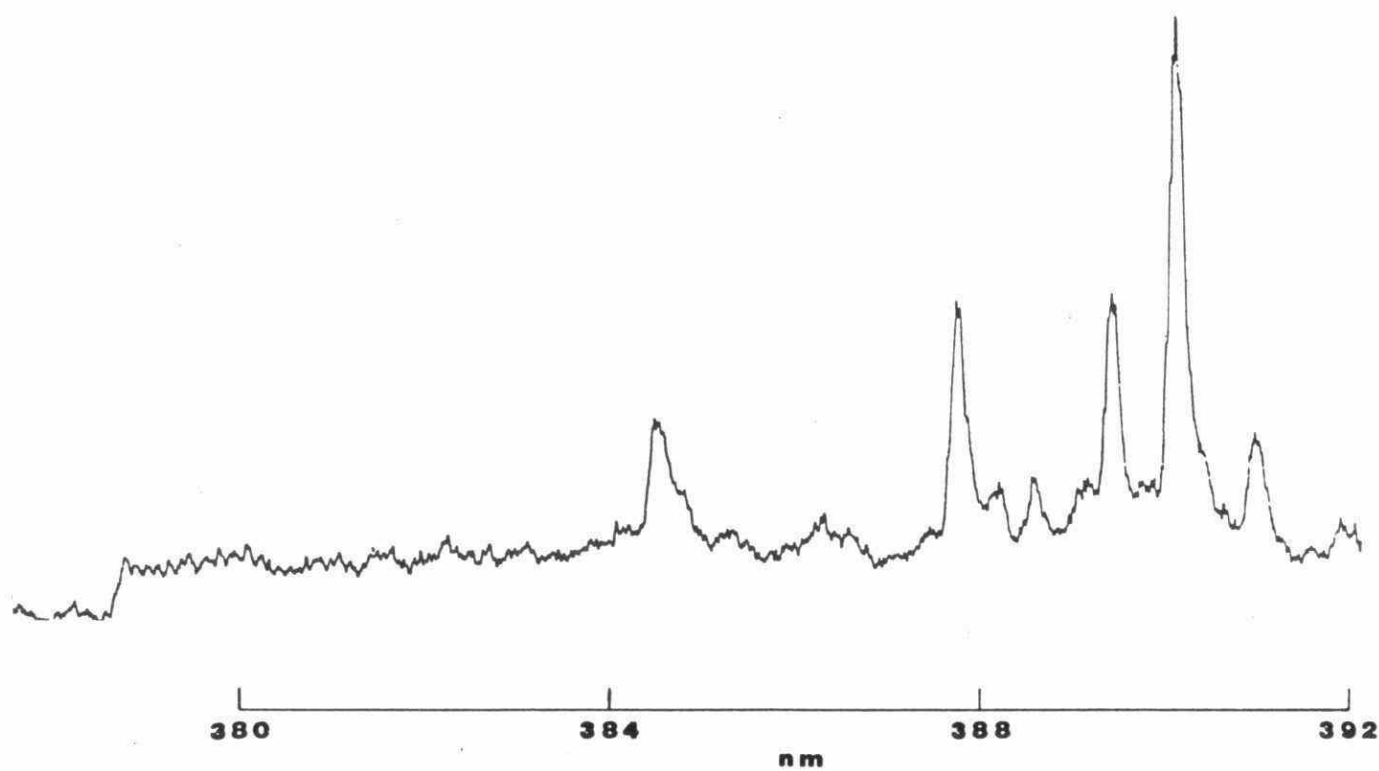
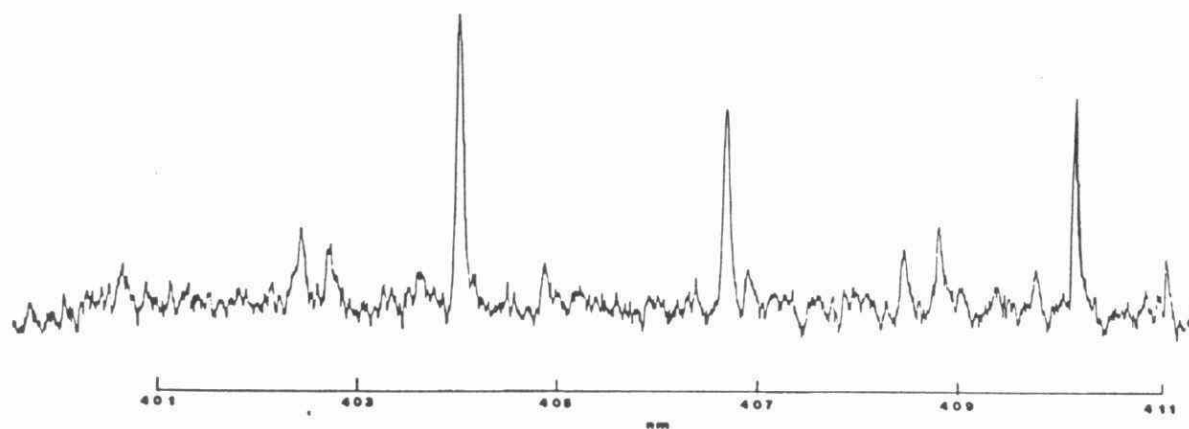


FIGURE 9: Jet-Cooled Laser Excitation Spectrum of Pyrene

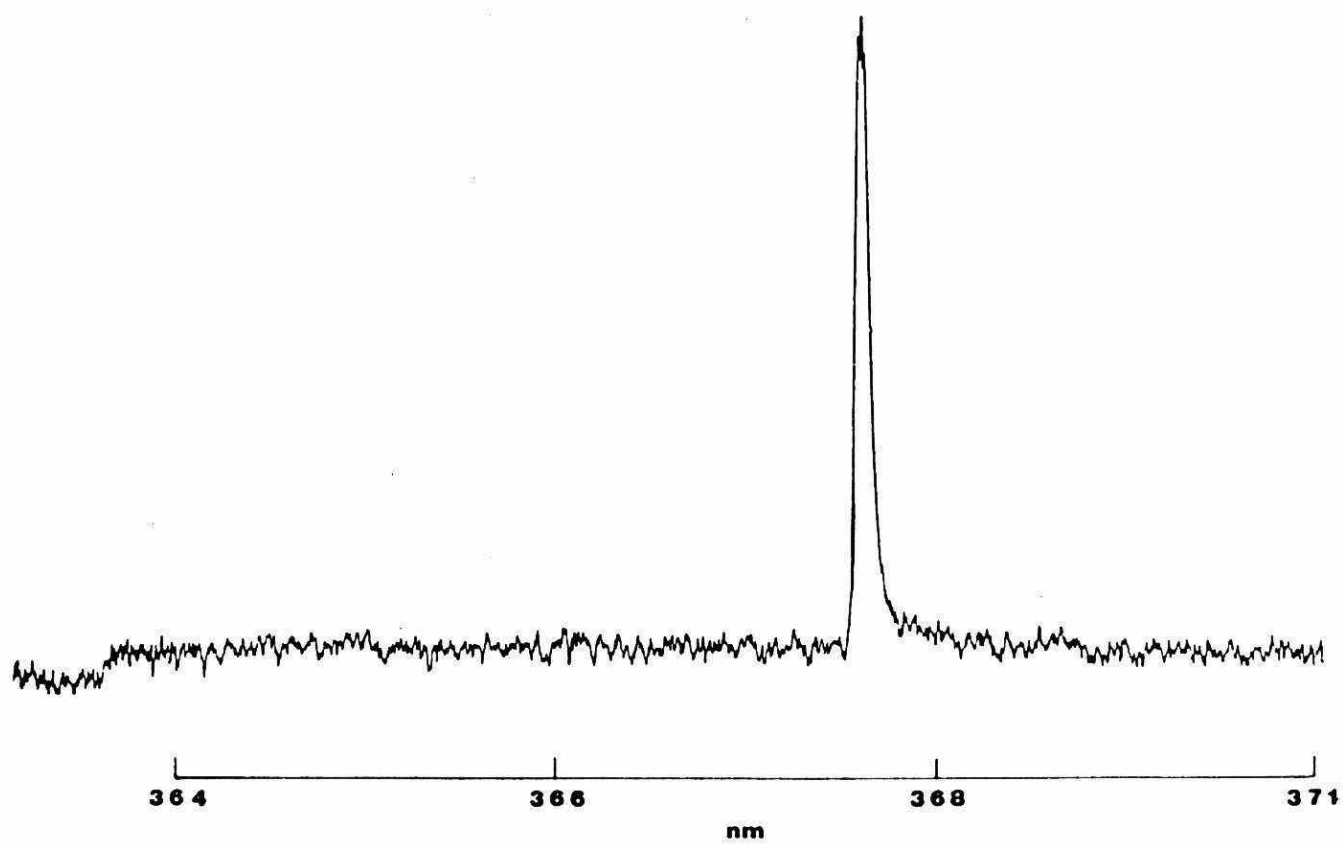


FIGURE 10: Jet-Cooled Laser Excitation Spectrum of Perylene

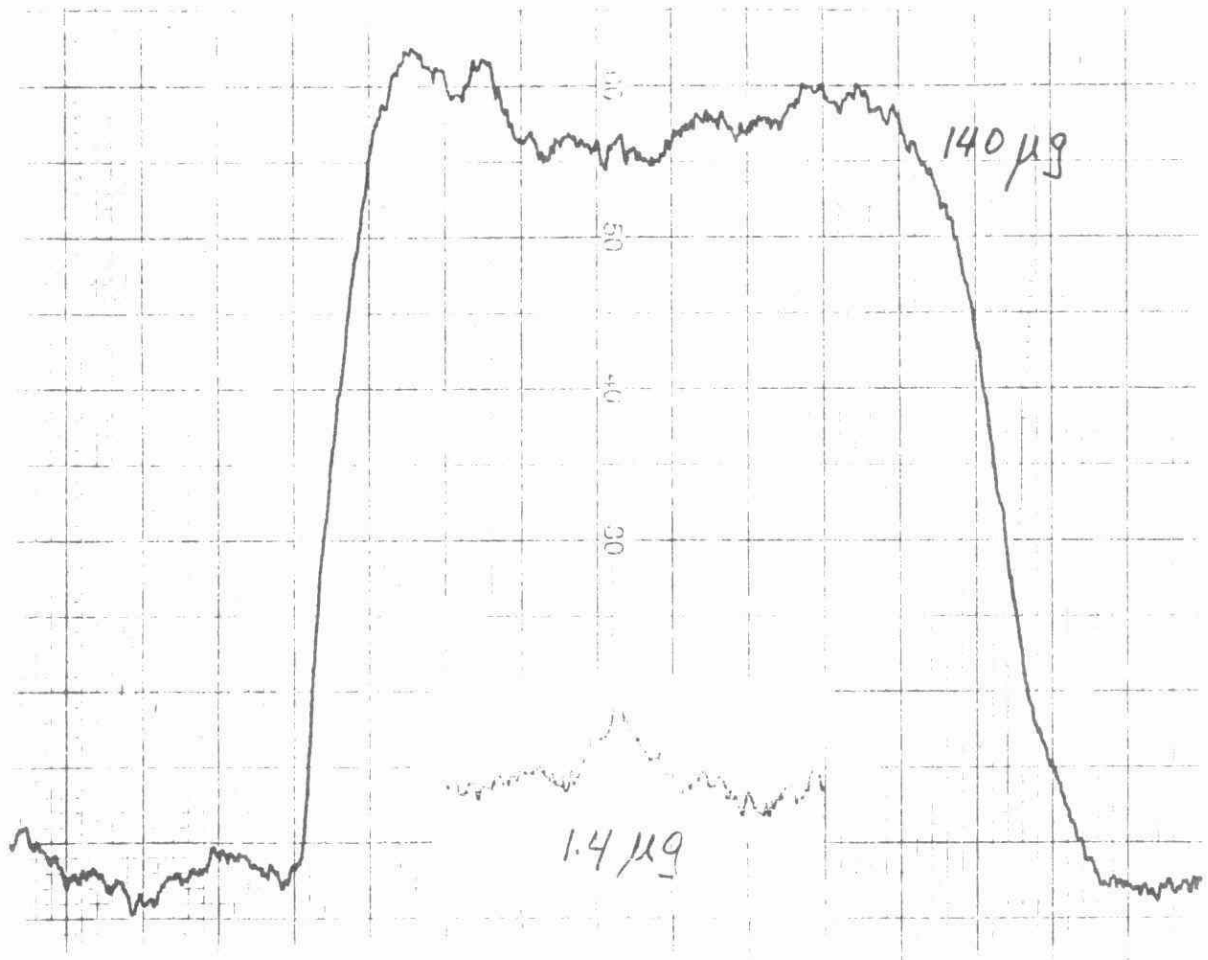


FIGURE 11: Laser Excited Fluorescence Signal vs Time
for Pyrene in a Supersonic Jet of Argon

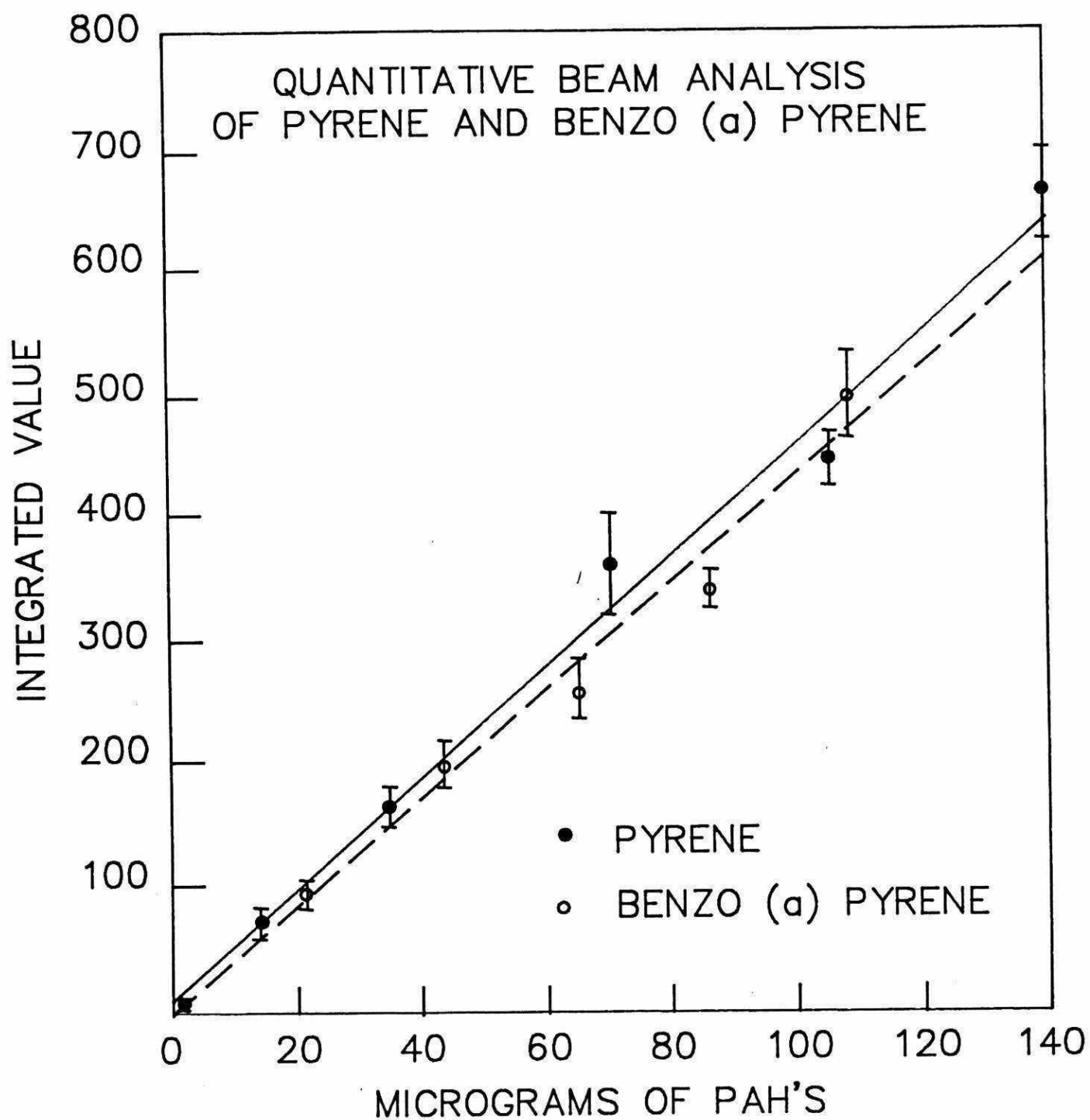


FIGURE 12: Plots of Time-Integrated Fluorescence Signals vs Mass of Sample from Quantitative Beam Analysis

NEW GENERAL SYNTHETIC METHODOLOGY FOR EXPEDIENT PROVISION OF
ISOMERICALLY PURE PAH ANALYTICAL STANDARDS

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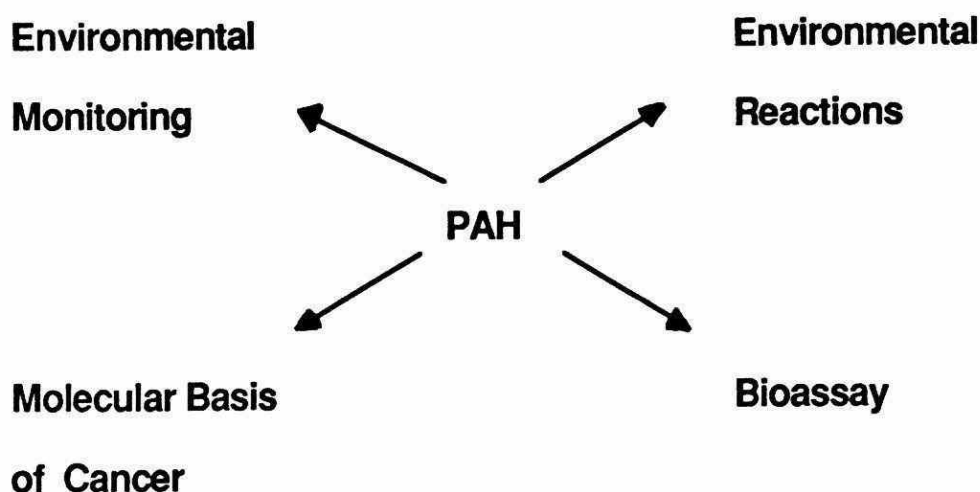
INTRODUCTION

Polycyclic aromatic hydrocarbons constitute a major class of organic pollutants widely dispersed in the environment arising from the incomplete combustion of fossil fuels and other organic matter (1). Human exposure to PAH through tobacco smoke and in certain occupations and urban environments has led to the strong inference that these compounds are important cancer causative agents in man (2) and thus has led to extensive research into the formation, occurrence, detection, chemistry, and metabolism of PAH (3). Interest in the corresponding aza-PAH is of more recent origin, having been stimulated by their discovery in samples of particulate matter, street dust, automotive exhausts, lake sediments, and cigarette smoke (1b, 4). In view of evidence of their carcinogenic nature, aza-PAH have prompted systematic characterization and biological testing as well (5). Methyl-PAH are always components of environmental PAH samples and recent studies have demonstrated that methyl substitution confers a remarkable enhancement in the tumorigenicity of PAH. Finally, the discovery of potent direct acting mutagenicity of nitro-PAH in 1980 and the accumulating evidence of their wide distribution resulting from diesel exhaust, fly ash, photocopier fluids, and cigarette smoke has resulted in increased research in the detection and elucidation of biological activity of this class of compounds (8).

Recent studies have demonstrated that the carcinogenic activity of PAH is expressed via prior metabolic activation (2b, 9). The primary metabolite arene oxides (proximate carcinogens), formed by enzymatic addition of an atom of oxygen, are relatively unstable and are further modified to dihydrodiol epoxides (ultimate carcinogens) which covalently modify DNA (10). The Bay Region theory of carcinogenesis suggests that dihydrodiol epoxides of bay regions of PAH are important for the initiation of cancer (9, 10, 11), although it cannot explain the effects of methyl substitution on carcinogenic activity (12).

Organic synthesis is the *sine qua non* condition of all PAH research. PAH compounds are required in high analytical purity for studies concerning a) environmental monitoring of their fate and distribution, b) monitoring of their environmental reactions (e.g. with ozone, NO_x), c) comprehensive understanding of their metabolic conversion to the ultimate carcinogens and therefore insight into the molecular basis of cancer and d) bioassay of newly discovered PAH to establish mutagenicity/carcinogenicity levels to warn of potential danger (Figure 1) .

Figure 1



Recent work in our laboratories has led to the development of a new general methodology for the synthesis of a variety of PAH and aza-PAH of environmental interest based on the aromatic directed metalation strategy (**Figure 2**) (13). Extension of this tactic has also allowed the preparation of previously inaccessible halo- and methyl-PAH involving regiospecific silicon protection of aromatic C-H and C-methyl sites (**Figures 3,4, 5, and 6**). Herein we review our work and new results connecting the directed metalation strategy with other modern synthetic methodologies: a) silicon activation for the preparation of nitro-PAH (**Figure 7**), b) silicon activation for regiospecific synthesis of indanols (**Figure 9**), and c) transition metal catalyzed cross coupling reactions leading to nitro-biphenyls (**Figure 10**). These reactions constitute prototypes of new methodologies promising to provide PAH and nitro-PAH by economic, efficient, and regioselective routes which avoid problems of isomer separation and excessive handling of carcinogenic substances as are normally associated with the available classical methods.

IMPROVED PREPARATION OF PAH-QUINONES AND CORRESPONDING PAH

As a direct consequence of general approaches towards the preparation of anthraquinones **2** and acridones **3** using ortho lithiated benzamides **1** (**Figure 2**), a number of PAH-quinones and PAH-heterocyclic quinones were prepared (14). A general, one-pot procedure was developed involving metalation (sec-BuLi), treatment with aromatic aldehydes, metalation, and aerial oxidative workup. Subsequent mechanistic investigation (15) led to the suggestion that excess base would have a favorable effect on the yields of this reaction and a new procedure was developed involving metalation with four equivalents of sec-BuLi followed by Jones oxidation. Using this procedure, a number of PAH-quinones were obtained

in significantly improved yields compared to those achieved previously (15) (**Table 1**). Although the scope of this method has not been exhaustively evaluated, its limitations will most likely arise in reactions of heterocyclic systems (e.g. pyridine) which are highly susceptible nucleophilic attack by reagents. Further mechanistic investigations currently in progress (16) may shed more light on the potential of this short synthesis of PAH-quinones.

Since PAH-quinone to corresponding PAH conversion is a standard, well-documented reaction (17), the overall route represents an efficient regimen which competes favorably with reported classical syntheses of certain PAH compounds (18). To demonstrate this, we have converted several of the PAH-quinones to the PAH (e.g. benz[a]anthracene, dibenz[a,c]anthracene, and dibenz[a,h]anthracene) using the aluminum tricyclohexoxide in cyclohexanol method (19).

REGIOSPECIFIC ROUTES TO PERI-METHYL-PAH AND AZA-PAH USING SILICON PROTECTION PROTOCOL

Although Newman has carried out extensive systematic work concerning the synthesis of monomethylbenz[a]anthracenes (20), many methyl-PAH are poorly accessible owing to the complexity of existing synthetic methods. Recent work in our laboratories has demonstrated that the trimethylsilyl group functions effectively to mask a) the more reactive metalation site ortho to a carboxamide and b) the ortho methyl moiety in an ortho-toluamide, thus allowing metalation and electrophile introduction into the alternate ortho positions (21). Since silicon groups can be easily introduced (RLi/TMSCl) and removed (CsF or tetra n-butyl ammonium fluoride (TBAF)), this method allows rapid entry into new diversely functionalized aromatic systems (21).

Following work concerned with the development of methodology and its application to the synthesis of naturally occurring anthraquinones (22), we focussed on its utility for the synthesis of peri-methyl PAH (**Figure 3**) (23). Thus treatment of the bis-trimethylsilyl *o*-toluamide (**4**) under standard metalation conditions followed by sequential reaction with 2-naphthaldehyde, CsF, and *p*-toluenesulfonic acid (TsOH) afforded, without purification of intermediates, the phthalide **5** in 52% overall yield. Taking advantage of techniques previously developed in our laboratories (22), the intermediate **5** was hydrogenolized, cyclized and oxidized to give 11-methyl-7,12-benz[*a*]anthraquinone **6** in 31% overall yield based on **4**. Since the conversion of **6** into 7,11,2-trimethyl-benz[*a*]-anthracene has been previously achieved, this constitutes a rapid and efficient route to this highly carcinogenic substance. Using identical sequences of reactions, the preparation of the 8-methyl-7,12-benz[*a*]anthraquinone and 10-methyl-9,14-dibenz[*a,c*]anthraquinone from 2-naphthaldehyde and 9-phenanthrene carboxaldehyde respectively was achieved in good overall yields (23). These quinones are also immediate precursors to the corresponding methyl-PAH by known reductive methods (19).

We envisage that the combination of our new method of ortho *N*-arylation (24) coupled with the silicon protection tactic would provide new routes to methyl substituted acridones and hence the corresponding aza-PAH. As an exploratory experiment investigated recently, the lithiated species **7** (**Figure 4**) was treated with the anilido-cyano cuprate **8**. Following oxygenation and the standard desilylation, the *o*-methyl *N*-arylanthranilamide **9** was obtained in 48% yield. Direct Friedel-Crafts cyclization using heptafluorobutyric acid (HFBA) afforded 1-methyl acridone **10** in good overall yield. Since the reduction of acridones into the corresponding acridines is a well established procedure (25), the overall route constitutes a general route to a number of environmentally significant aza-PAH.

HALO-PAH USING SILYLATED BENZAMIDE INTERMEDIATES

In view of the widespread occurrence of chloroaromatic pollutants (PCBs, dioxins, chlorobenzene) and their continued incineration (26), interest in the detection and analysis of chloro-PAH and halo-PAH in general has increased significantly in recent years(27). Chloro-PAH have been investigated to determine the effect of chloro substitution on the metabolism of PAH (28) while bromo-PAH have been detected in gasoline and diesel fueled vehicle emissions. Structure-carcinogenic activity correlation studies on bromo-PAH and fluoro-PAH have been reported (30) and the latter class has been used for 'markers' to establish the site of metabolic activation (31).

As an extension of the use of silicon protection protocol in the context of the directed ortho metalation reaction described above, we developed regiospecific routes to several bromo-, chloro-, and fluoro-PAH quinones (32, 33). The readily available 2,6-dibromobenzamide **11** (**Figure 5**) was subjected to monometal-halogen exchange followed by treatment with either 1-naphthaldehyde or 2-naphthaldehyde, and the crude products derived were cyclized with TsOH in refluxing toluene to afford the phthalides **12** and **13** in 67% and 78% overall yields respectively. Slight modification (32) of the regimen described above for the preparation of methyl-PAH (**Figure 3**) led, in several steps, to the bromo benz[a]anthraquinones **14** and **15** which, as in the previous anthraquinone examples, could be converted into the bromo-PAH and dimethylbromo-PAH using standard methodology developed by Newman (30).

Similarly, the highly condensed bromo-PAH quinones **16** and **17** were prepared by quenching the monolithio species derived from **11** with either 9-phenanthrene carboxaldehyde or 1-pyrene carboxaldehyde respectively and following the standard phthalide to anthraquinone procedure described above for methyl-PAH.

The knowledge that meta-related chloro or fluoro and carboxamide substituents undergo regiospecific ortho metalation in the site common to both (34) allowed the synthesis of the corresponding chloro- and fluoro-anthraquinones using the silicon protection concept (**Figure 6**) (33). Thus standard metalation and silylation of **18**, X = C, F, smoothly led to the formation of the respective silylated products **19** which upon further metalation and condensation with benzaldehyde, and TsOH cyclization furnished the chloro- and fluoro-phthalides whose conversion into the corresponding anthraquinones **21** followed the **procedures established for the preparation of the bromo-PAH quinones described above**. These examples demonstrated the utility of metalation-silylation strategies for convenient access to certain halo-PAH in high yields and short order. The potential for synthesis of more highly condensed halo-PAH appears to be promising.

SILYLATED PAH QUINONES AS INTERMEDIATES FOR NITRO-PAH

Cognizance of the facile ipso nitrodesilylation of aryl silanes (35, 36) suggested that silylated anthraquinones **24** may serve as potential intermediates for the corresponding nitro-PAH systems (**Figure 7**). To achieve this end, the ortho silylated benzamide **22** was metalated, treated with 2-naphthaldehyde, and cyclized with TsOH to give the silylated phthalide **23** in good overall yield. The conversion of **23** into the corresponding anthraquinone **24** and thence into the nitro-PAH, **25**, currently in progress, may necessitate the prior reduction of the quinone moiety in order to activate the ipso nitrodesilylation process (35, 36). The concomitant reduction of the nitro group, if it occurs, will be redressed by a reoxidation reaction (37).

A GENERAL 1-INDANOL SYNTHESIS VIA FLUORIDE-INDUCED INTRAMOLECULAR CARBODESILYLATION. POTENTIAL APPLICATION TO FLUORENES

Although the classical Friedel-Crafts reaction is a well-travelled route for annelation of 5-, 6-, and 7-membered ring ketones to aromatic substrates (38), its regiospecificity is dictated by the normal electrophilic substitution rules which preclude the preparation of certain substituted systems without resorting to protection divergencies and multi-step regimens. A case in point is the intramolecular Friedel-Crafts reaction of methoxy-substituted **26** (Figure 8) which invariably leads to para- (27) and not the desired ortho-substituted product. As a result of our interest in connecting arylsilane derived methodology to the directed metalation reaction and the recent demonstration by Effenberger of fluoride-induced ipso carbodesilylation in reactions of arylsilanes with aromatic aldehydes (39), we sought to devise new routes to 7-methoxy-1-indanols **34** (Figure 9), synthons which are of demonstrated utility a number of applications and of potential use for the preparation of nitro-fluorene PAH.

To derive the key intermediates (Figure 9), the silylated meta-anisamide **29** was sequentially treated with diborane and vinyl chloroformate (40) to afford the unstable benzyl chloride **30** which, according to the excellent procedure of Kozlewski (41), was smoothly converted into the benzaldehyde **31**. Wadsworth-Emmons-Horner reaction followed by hydrogenation gave the phenyl propionate **32**, $R^1 = H$, $R^2 = Me$, whose conversion into the corresponding aldehyde **33** was achieved by standard diisobutylaluminum hydride (dibal) reduction. The methyl substituted aldehyde **33**, $R^1 = Me$, $R^2 = H$, was similarly obtained while the corresponding dimethyl derivative **33**, $R^1 = R^2 = Me$, was prepared by a different route (42).

When the three aldehydes **33** were subjected to reaction with CsF in anhydrous DMF, the 1-indanols **34** were obtained in 50-70% yields. Since the highest yields are obtained on **33**, $R^1 = R^2 = H$, the most likely system to undergo fluoride-catalyzed aldolization (43), the yield appears not to be reflective of such a process.

To test the possibility of an intramolecular electrophile-induced ipso desilylation, a well-established method without sufficient synthetic application (36), the acid chloride corresponding to the ester **32**, $R = H$, readily obtained by standard base hydrolysis followed by oxalyl chloride treatment, was subjected to reaction with various Lewis acids ($AlCl_3$, $TiCl_3$, $SnCl_4$). In all cases, 5-methoxy-1-indanone, the product of desilylation and normal Friedel-Crafts acylation, was obtained. This result, presumably due to steric factors in the formation of the Lewis-acid coordinated ipso intermediate, mitigates the potential use of the acylium ion induced carbodesilylation process.

In summary, the fluoride-induced intramolecular carbo-desilylation reaction, **33**→**34** is a useful procedure for the efficient access to 7-methoxy-1-indanols. The application of this method for the construction of fluorene-PAH is currently under investigation in our laboratories.

A NEW NITRO-H PROTOTYPE CONSTRUCTION BASED ON Pd-CATALYZED CROSS COUPLING OF BORONIC ACIDS WITH ARYL BROMIDES

The increasing evidence that nitro-PAH is a widely distributed, highly mutagenic class of environmental pollutants (7) has stimulated analytical and biological research in this area (8). In support of these studies, synthesis and complete characterization of nitro-PAH is a prime requirement. The currently available methods are invariably based on electrophilic substitution

and other classical reactions which suffer from low yields, lack of isomeric specificity, and limited scope (8).

We have recently developed a new general method for the construction of unsymmetrical biaryls and heterobiaryls with diverse functionality using a transition metal catalyzed cross coupling reaction of aryl boronic acids with aryl bromides (44). We are in the process of expanding this method to include the preparation of nitro-PAH including nitro-fluorenes(nitro-F), nitro-fluoranthenes(nitro-FA), and nitro-phenanthrenes (nitro-P) (45). The preliminary methodological results are encouraging and these, together with application for the preparation of nitro-F are described here.

Following the development of our general protocol (**Figure 10**) (44), we have carried out the cross coupling reaction between the ortho boronic acid benzamide **36**, prepared from the parent benzamides **35** and aryl bromides (ArBr) containing nitro functionality (ortho, meta, para-nitro bromobenzenes have been used). In all cases, high yields of the respective coupling products **37** (Ar = nitro-aromatic) have been obtained.

These successful results provide the impetus to undertake the preparation of nitro-F PAH substances (**Figure 11**). The isomeric NO₂-ortho-bromobenzaldehydes **38**, all of which are available by classical syntheses, will be coupled with phenylboronic acid **39** to give the NO₂-biphenyl aldehydes **40**. The conversion of **40** into the NO₂-F **43** will be carried out in two sequences: either borohydride reduction followed by precedent (46) acid-catalyzed cyclization or oxidation to the carboxylic acid **41** followed by the more classical Friedel-Crafts reaction to the intermediate fluorenone and reduction to the nitro-F (42). Using this prototype sequence of reactions, the synthesis of a variety of nitro-F derivatives (Table 2) and in, projection, the corresponding nitro-fluoranthenes will be feasible.

CONCLUSIONS

The synthesis of a number of PAH, aza-PAH, methyl-PAH, halo-PAH have been achieved by routes which are short, efficient, and avoid excessive contact with intermediates and carcinogenic products. In addition, key synthons have been constructed which will allow the preparation of new methyl aza-PAH, methyl halo-PAH, and several classes of nitro-PAH including nitro-fluorenes, nitro-fluoranthenes, nitro-phenanthrenes, and nitro-benz[a] anthracenes. A sizeable repository of PAH samples are currently available in high purity and new PAH are being synthesized on a continuing basis which will provide the necessary analytical standards for present or future, possibly unanticipated, environmental problems.

ACKNOWLEDGEMENT

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Figure 2

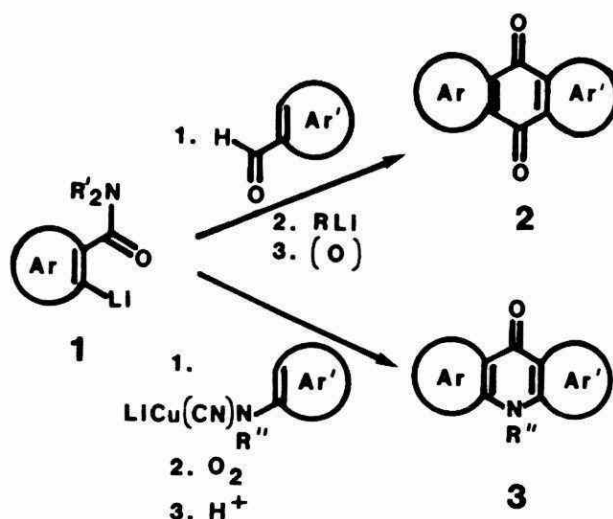


Table 1. PAHs, PAH-Quinones and Aza-PAHs Synthesized in Our Laboratories

PAHs (and Precursor Quinones)

anthracene-9,10-dione
 1-methoxyanthracene-9,20-dione
 2-methoxyanthracene-9,10-dione
 benz[a]anthracene-7,12-dione
 benz[b]anthracene-5,12-dione
 dibenz[a,c]anthracene-9,14-dione
 dibenz[a,h]anthracene-7,14-dione
 dibenz[a,i]anthracene-7,14-dione
 dibenz[b,i]anthracene-6,13-dione
 naphtho[2,3-a]pyrene-9,14-dione
 8-methyl-benz[a]anthracene-7,12-dione
 11-methyl-benz[a]anthracene-7,12-dione
 13-methyl-dibenz[a,c]anthracene-9,14-dione
 benz[a]anthracene(BA)
 benz[b]naphthacene
 dibenz[a,c]anthracene
 dibenz[a,h]anthracene
 8-bromo-7,12-benz[a]anthraquinone
 11-bromo-7,12-benz[a]anthraquinone
 10-bromo-9,14-dibenz[a,c]anthraquinone
 3'-bromonaphth-(2',3':3,4)-pyrene-1',4'-quinone

Aza-PAHs

benz[a]acridine
 benz[c]acridine

Synthesis of Peri-Methyl Substituted PAHs

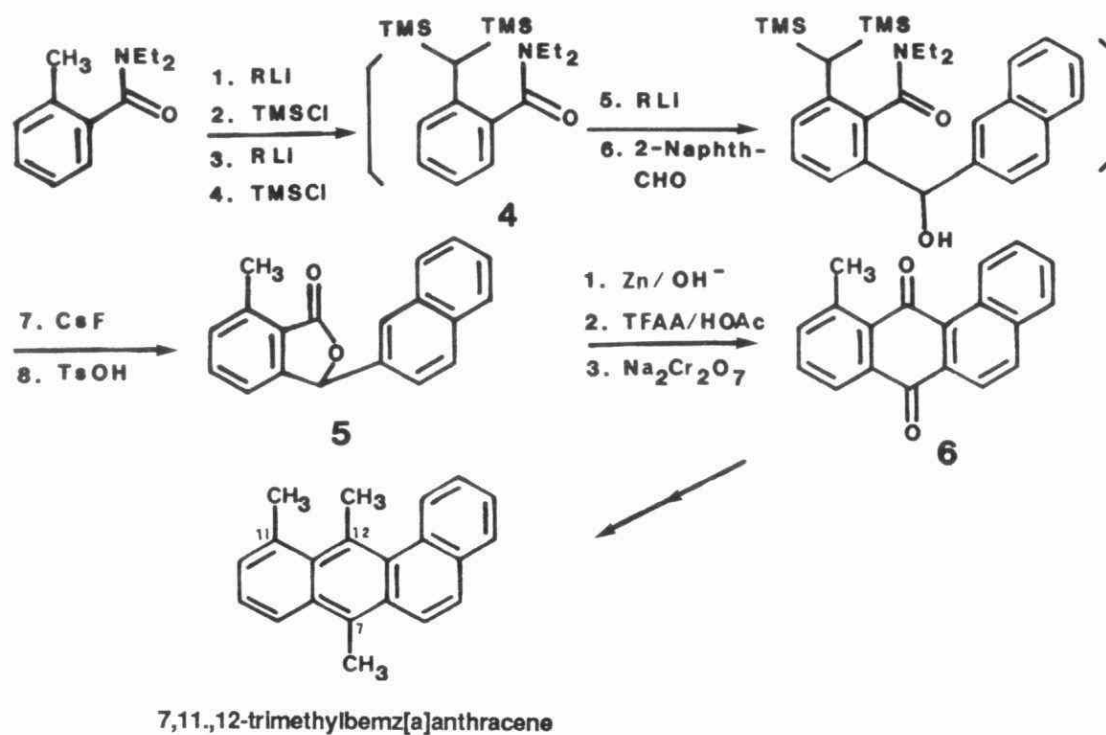
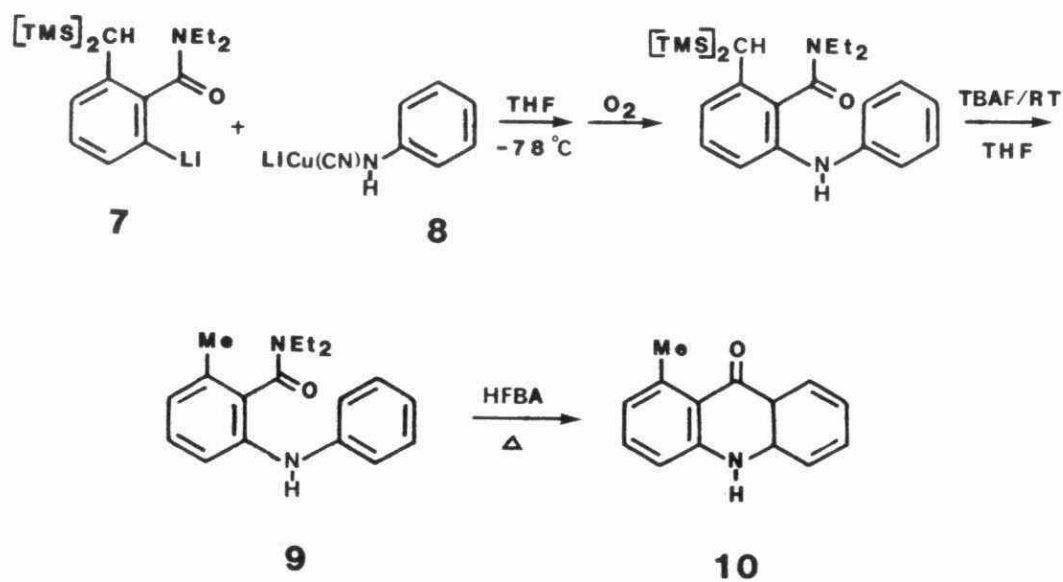


Figure 4



Synthesis of Bromo Substituted PAHs

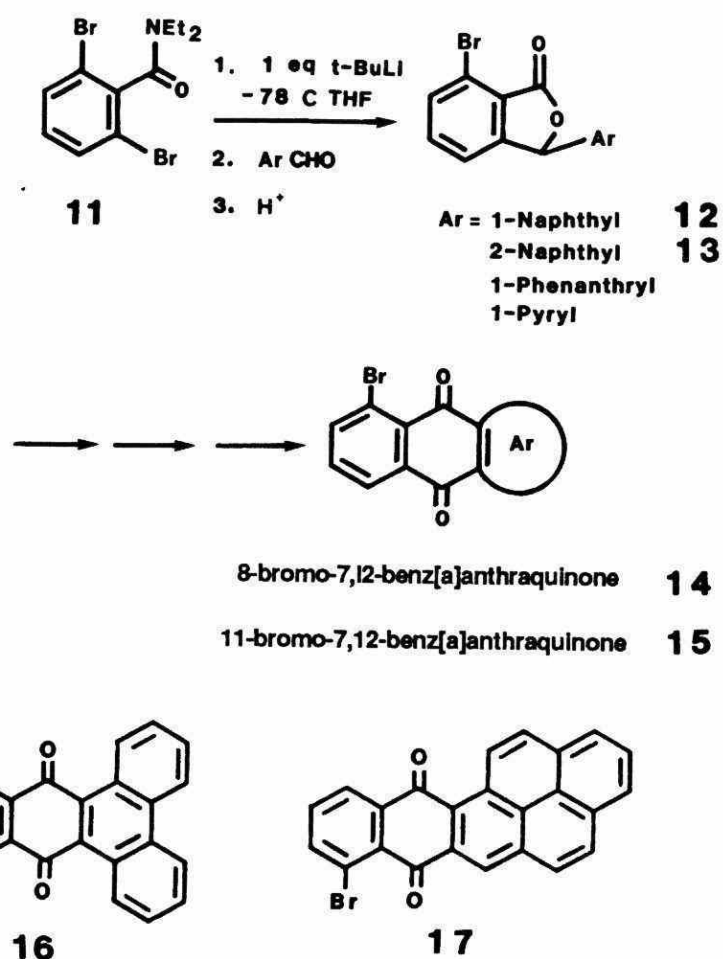


Figure 6

Synthesis of Chloro- and Fluoro-PAHs

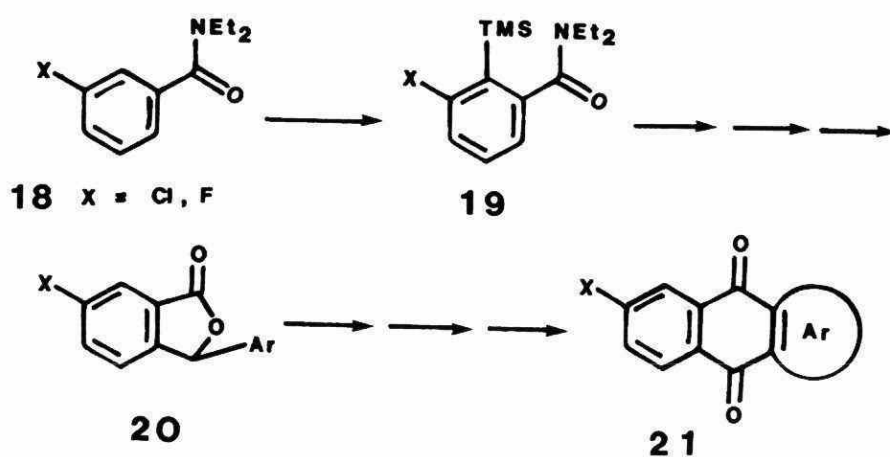


Figure 7

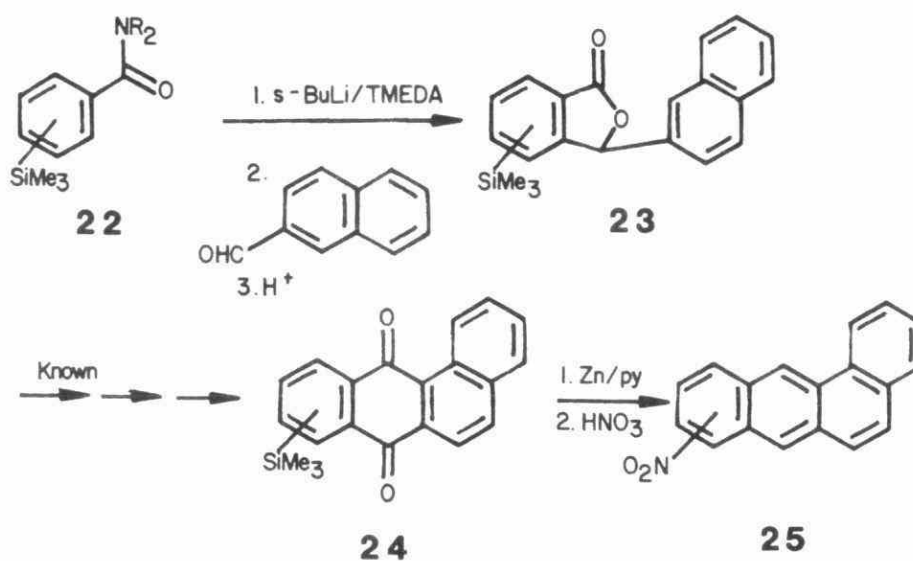


Figure 8

Aromatic Electrophillic Substitution

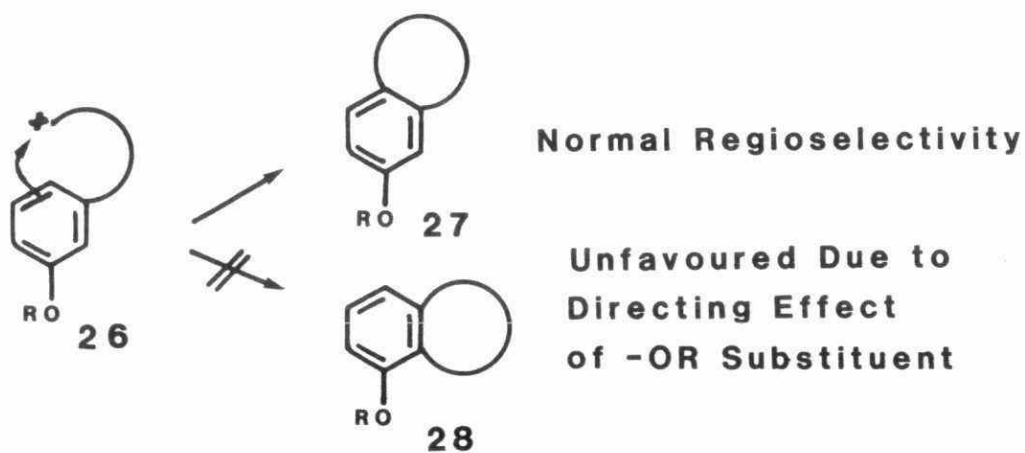


Figure 9

Silicon-Directed Synthesis of 7-Methoxy-1-Indanols

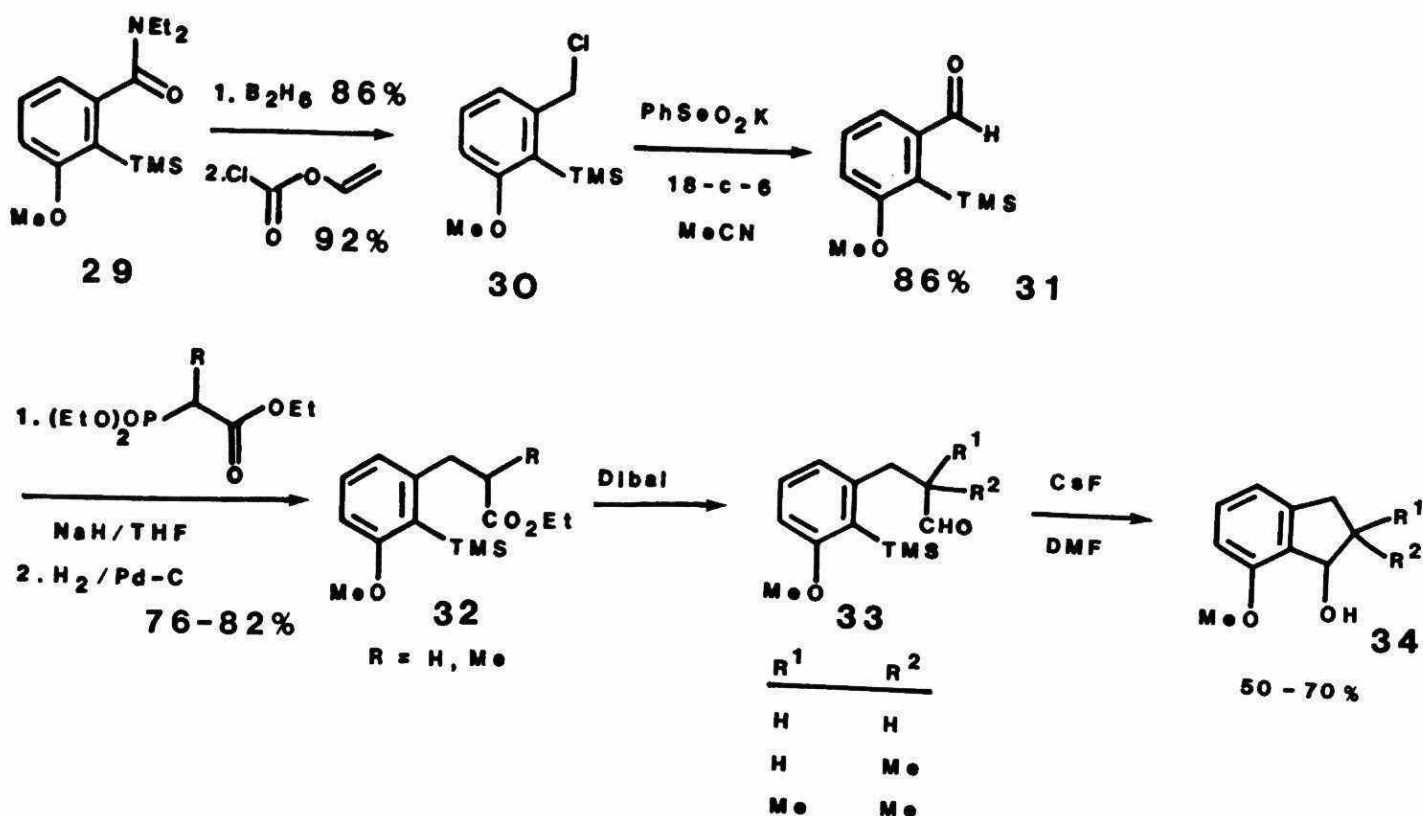


Figure 10

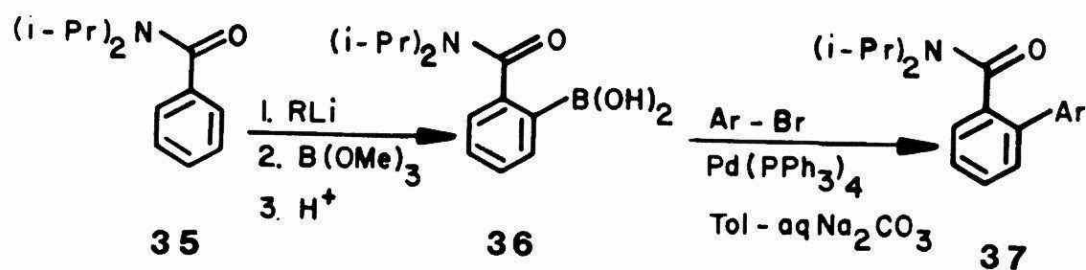


Figure 11

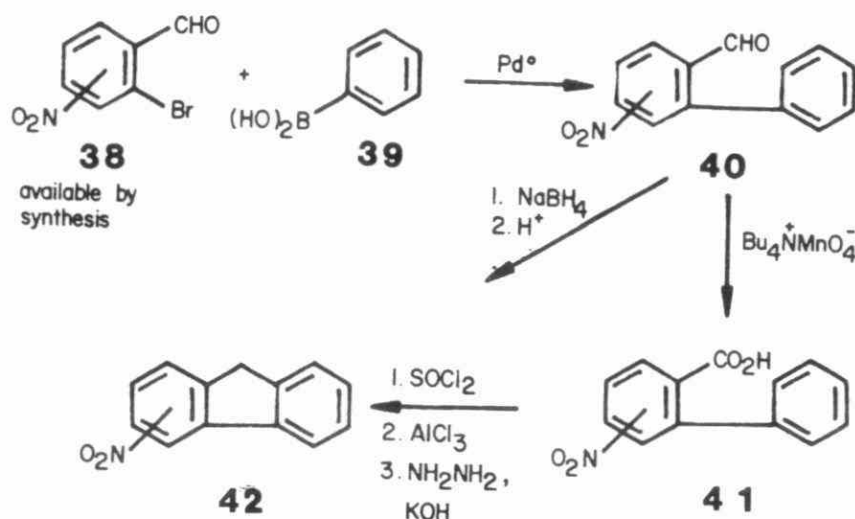
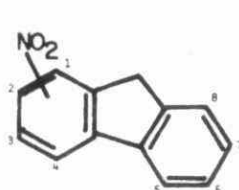


Table 2. Provision of Nitrofluorenes and Nitrofluoranthenes and their Hydroxylated Derivatives.

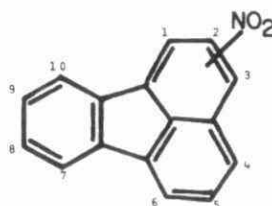


Nitrofluorene(F)

- 1-NO₂-F
- 2-NO₂-F
- 3-NO₂-F
- 4-NO₂-F

Metabolites/Oxid. Products:

- 1-NO₂-5-OH-F
- 2-NO₂-5-OH-F
- 3-NO₂-5-OH-F
- 4-NO₂-5-OH-F
- 1-NO₂-6-OH-F*
- 2-NO₂-6-OH-F*
- 3-NO₂-6-OH-F*
- 4-NO₂-6-OH-F*
- 1-NO₂-7-OH-F
- 2-NO₂-7-OH-F
- 3-NO₂-7-OH-F
- 4-NO₂-7-OH-F



Nitrofluoranthene (FA)

- 1-NO₂-FA
- 2-NO₂-FA
- 3-NO₂-FA
- 7-NO₂-FA
- 8-NO₂-FA
- 9-NO₂-FA
- 10-NO₂-FA
- 3,7-diNO₂-FA
- 3,8-diNO₂-FA
- 3,9-diNO₂-FA
- 3,10-diNO₂-FA

Metabolites/Oxid. Products:

- 3-NO₂-6-OH-FA
- 3-NO₂-8-OH-FA(?)
- All isomeric 1-, 2-, 3-, and 4-NO₂-FA with 5-, 6-, and 7-OH available from corresponding NO₂-OMe-F precursors.

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